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STAIN TECHNOLOGY

VOLUME 26 JULY, 1951 NUMBER 3

OBTAINING UNIFORM RESULTS IN THE HISTOCHEMICAL TECHNIC FOR ACID PHOSPHATASE[1,2]

JOHN B. GOETSCH[3], and PATRICIA M. REYNOLDS, *Yale University School of Medicine, Department of Surgery Section of Urology and the Department of Pathology*

Received for publication Sept. 9, 1950

ABSTRACT.—**The capricious nature of the results obtained in acid phosphatase preparations is notorious. An investigation of the causes of this is presented. It was found that it is possible to produce uniform preparations. In addition to many small details which must be carefully followed, two major factors radically affecting the results were observed: the technic of mounting the section on the glass slide, and the technic of deparaffinization and hydration. The final technic worked out is presented in the Summary and Conclusions.**

I. INTRODUCTION

In a study of the distribution of the acid-phosphatase activity in fixed tissue sections, it became apparent that the lack of uniformity in the preparations severely limited the value of such an investigation. This difficulty is a common experience and is mentioned in almost every paper which has appeared on the subject. It has been demonstrated by chemical means repeatedly that there is a major loss of acid-phosphatase activity in tissue when it is fixed and prepared for sectioning and staining[4]; however, this would not explain the variation from one block to another from the same tissue source, or the irregularities noted in adjacent serial sections on the same slide or indeed from one area to another in the same tissue section. It is the purpose of this paper to report results of a study of this problem and to outline the precautions which must be observed in order to obtain uniformity in preparations for the microscopic localization of acid-phosphatase activity.

---

[1]Supported by a grant from the National Cancer Institutes of the United States Public Health Service.

[2]Much of this material was presented by one of us (J.B.G.) at the meeting of the Histochemical Society, March 25, 1950, held in Philadelphia.

[3]This work was begun while a fellow of the Jane Coffin Childs Fund.

[4]See Stafford and Atkinson (1948), Doyle (1948), Rabinovitch *et al.* (1949).

II. Methods

The lead nitrate method for demonstration of acid-phosphatase activity devised by Gomori (1941) was the method used throughout. This consists in incubating sections of acetone-fixed material in an acetic acid, sodium acetate buffer to which lead nitrate and sodium glycerophosphate have been added. The enzyme liberates phosphate ions which combine with lead ions precipitating lead phosphate at the site of enzyme activity. The white lead phosphate is converted to the more readily visible black lead sulphide by hydrogen sulfide. This is accomplished by bathing the sections in a weak solution of ammonium sulphide.

The standard technic of Gomori was used as a comparative base line. In each experiment only one step of the technic was varied. Sodium glycerophosphate was used as a substrate. A pH of 4.3–4.4 determined by glass electrode was used throughout.

Rat liver and kidney were the tissues generally used. Rat spleen, rabbit liver, kidney and spleen, human prostate, and prostatic carcinoma and benign prostatic overgrowth were used in some parallel experiments. No advantage of one tissue over another was noted other than the observation that human prostatic tissue contained so much enzyme that minor differences in results were often difficult to ascertain.

The results of varying each step in the procedure are here presented as well as the method finally recommended.

III. Conclusions

A. *Fixation:* There is general agreement among all investigators that absolute acetone is the best fixative for preservation of acid-phosphatase activity in fixed tissues. Fixation of tissues at 4°C. or 20°C. did not materially vary the results. The optimum length of exposure to acetone was found to lie between 8 and 24 hours. Tissues exposed beyond this time showed definitely less activity. The manner in which the tissues were exposed to acetone was found to be of greater importance than the time or temperature of exposure. Tissue blocks over 3 mm. in thickness were poorly fixed in their central portions especially if the capsule of the organ formed one or more surfaces of the specimen (i.e. liver, spleen, etc.). This was particularly true in relatively solid organs such as spleen and lymph node in contrast to kidney, for example, where circulation of the fixative through channels such as the tubules was possible. The volume of fixative used had to be sufficient to dehydrate the tissue completely, and free circulation of the fixative around the block was of definite advantage.

The freezing-drying technic produced excellent and uniform results. The acetone-fixed material showed only slightly more than half of the activity of the frozen-dried material as judged visually. Subsequent exposure of frozen-dried material to acetone decreased

its enzyme activity; however, the sites of localization of the activity were the same whether frozen-dried or acetone-fixed.

B. *Dehydration:* In studying the results obtained in repeated experiments, it became apparent that a short exposure (3–4 hours) to absolute alcohol had relatively little effect on the acid-phosphatase activity *provided* the tissue had been previously completely dehydrated. Since adequate dehydration had already been obtained by exposing the tissue to acetone alone, however, the subsequent step of exposure to absolute alcohol was discarded as not necessary.

C. *Clearing:* It was found that both toluene and benzene were very satisfactory clearing agents and that storage in them for 24 hours was not harmful. In contrast chloroform was found to produce a definite decrease in the enzyme activity. Benzene was selected as the clearing agent of choice since its hardening effect was less than that of toluene.

D. *Embedding temperature:* No difference was noted in the activity exhibited by tissues embedded at 45°, 60°, or 65°C. for two hours.[5] Those similarly embedded either at 72° or 82°C. showed distinctly less activity. No difference was noted between tissue embedded at 60°C. *in vacuo* or at ordinary atmospheric pressure. The importance of thorough fixation and dehydration was demonstrated by the inactivation of enzyme that occurred at temperatures as low as 45°C. in central, poorly penetrated areas of thick blocks.

E. *Mounting:* Paraffin ribbons were flattened on a warm water bath for 4 minutes without loss of acid-phosphatase activity in the included tissue. Strict precautions were taken to keep the temperature of the bath at 38–40°C., a point just sufficient to soften the paraffin but not high enough to melt the paraffin. When the temperature was higher, there was marked loss of activity along the edges of the sections where the melting began.

The use of adhesive agents to hold the sections on the slide was found to be a very important factor in the production of irregular results. The adhesive agents tried were celloidin and albumin, the latter being a mixture of egg albumin and glycerin with or without traces of thymol added as a preservative. When celloidin was used, the mounted tissue section was deparaffinized, washed in absolute alcohol, and coated by dipping in a dilute solution of celloidin in equal parts of ether and absolute alcohol. The film of celloidin was found to insulate the tissue sufficiently from penetration of the substrate so that very slight variations in the thickness of the coating caused proportional variations in the density of the subsequent pre-

[5] A special mixture of low-melting paraffin, stearic acid, and bees wax was used for the 45°C. embedding. In control experiments no difference was noted between sections embedded in this wax mixture at 60° and sections embedded in ordinary paraffin of melting point 56°C. at 60°C.

cipitate in the underlying section. When tissue sections were mounted on a slide coated with the albumin-glycerin mixture, with or without added thymol, marked inactivation of the enzyme activity was noted. If the slide was coated with a purposely thick application of albumin, only the ridges or folds in the section which were not in actual contact with the albuminized slide surface showed any enzyme activity. With a very thin coat of albumin this could also be observed but to a less degree. All of the above artifacts were avoided when no adhesive agent was used. The sections remained secure on the glass slides if the preparations were sufficiently dried before deparaffinization and immersion in the substrate solution. There was no detectable difference noted between the slides dried in the open air and those placed in the calcium chloride dessicator. No difference in activity was apparent between slides dried for ¼ hour or for intervals up to 72 hours at room temperature. Overnight drying was more than adequate even at relatively high humidity.

F. *Deparaffinization and hydration of sections:* Until the effect of dilute alcohols on the enzyme was noted, it had been standard practice to deparaffinize tissue sections with toluene or benzene, and subsequently to hydrate them by an alcohol-water series. (See "B" Dehydration). In order to determine the magnitude of the effect of this step in the technic, tissue sections were treated in the following manner: A number of sections cut from blocks of kidney, liver, spleen, intestine, and prostate, were divided into three groups. One set of slides was deparaffinized and hydrated in the usual manner by passing through toluene, absolute alcohol, 95% alcohol, to water and substrate. The second group was similarly deparaffinized by passing it through toluene, but then to toluene-acetone equal parts, to acetone, directly to water and then substrate. The third group was *not* deparaffinized. The paraffin ribbon was left unaltered on the glass slide and placed directly in the substrate. After 2, 4, and 8 hour periods of incubation in the substrate the slides were washed in water and placed in a weak ammonium sulfide solution. They were again washed, and it was after this that the third group of slides was deparaffinized. This was accomplished after the usual dehydration in alcohol and during the clearing in toluene preparatory to mounting in synthetic resin (Hartman Leddon Co.). The results were striking. The sections which showed the least enzyme activity were those which had been deparaffinized and hydrated in the routine manner by passing them from toluene through the alcohols to water before incubation in substrate. The second group in which hydration had been accomplished by passing from acetone to water after deparaffinization showed distinctly more activity. The third group in which the paraffin had not been removed until after incubation in the substrate showed the greatest enzyme activity.

On examining the sections in detail another very striking dif-

ference between the three groups of sections was noted. The first two groups which had been deparaffinized and passed through alcohol or acetone before incubation showed dense staining of all the nuclei and a generalized distribution of a lighter brown color throughout the cytoplasm of the epithelial cells. The third group which was not deparaffinized until after incubation showed, in contrast, a very sharp localization of precipitate (i.e. activity) in certain parts of the cytoplasm or in extracellular structures and very little in the nuclei. In the cells of the renal tubules, for example, mitochondria were distinctly outlined by the precipitate and could be readily differentiated from the adjacent cytoplasm that possessed much less activity. There was sharp localization of activity in the bile canaliculi between the liver cells with little in the cytoplasm except as observed in the mitochondrial granules and almost none in the nuclei. Similar differences in distribution of enzyme activity were observed in other tissues. In brief it would appear that this was one of the most important steps to be observed in the technic. Since it can be shown that diffusible substances in solution can reach all parts of the cells in a non-deparaffinized ribbon, it would seem that much that has been taken for the sites of acid-phosphatase activity has largely been artifact produced by the technic as usually carried out. Somewhat similar observations in distribution of alkaline phosphatase activity in non-deparaffinized preparations have been made by Ryter and Neumann (1949). Further discussion of this will be presented in a future publication.

G. *Substrate:* The sodium glycerophosphate substrate recommended by Gomori was used exclusively.

A wide choice in the source of the sodium-glycerophosphate was possible, although information as to the relative amounts of α and β molecules was not available. Five samples from three different manufacturers were used and all gave identical results. A wide variation in concentration of sodium glycerophosphate in the substrate solution was found to yield similar results. No difference was detectable when substrates having only one third the usual amount of glycerophosphate were used. However, if the slide was not immersed in an ample volume of substrate or if the layer of substrate to which the slide surface was exposed was of uneven depth, the precipitation of lead phosphate at the sites of enzyme activity was not uniform over the slide: the amount of the precipitate in the tissues at one end of the slide was different from that at the other. The white precipitate of lead phosphate noted immediately upon the addition of lead nitrate in the preparation of the substrate can be minimized by using a freshly prepared glycerophosphate solution and by adding the lead nitrate as the last ingredient. The precipitate continues to form slowly even after filtration so that the slides become covered with fine granules of lead phosphate. This can be eliminated entirely by allowing the sub-

strate to stand overnight at room temperature before filtering. No matter how much precipitate formed during the preparation of the substrate, there was always a sufficient concentration of lead ions and glycerophosphate in solution so that the effectiveness of the substrate was unimpaired.

Investigation of the incubation temperature was carried out. Sections from the same blocks were incubated at 37°, 45°, and 60°C. for 2, 4, 6, 8, 10, 12, 14, and 24 hours. The tissue incubated at 37°C. and 45°C. showed progressive deposition of precipitate at the sites of enzyme activity during the entire period. The slides at 45°C. showed more rapid deposition than at 37°C. as might be expected. Those incubated at 60°C. showed even more rapid deposition of precipitate up through the first four hours but very slight, if any, further deposition from then on. It was apparent that an incubation temperature of 45°C. or less was perfectly safe but that the rehydrated enzyme was almost, if not completely, inactivated by more than a four-hour exposure to 60°C.

H. *Storage:* Sections cut from an acetone-fixed previously uncut block of human prostate stored in the refrigerator for six months, showed no detectable difference in activity when compared with sections cut from freshly embedded tissue; however, freshly cut sections from a block of human prostatic tissue that had originally been cut six months before and subsequently stored under the same conditions showed almost complete loss of enzyme activity.

By subdividing a paraffin block from which sections had been cut and then recutting the portions one at a time, it was shown that no significant decrease in the activity of the tissue took place for about eight days. After that time, however, there was rapid decrease of activity. Storage in the refrigerator slowed down the deterioration to a slight extent. Coating the cut surface of the blocks with paraffin after cutting the first sections had no appreciable effect in retarding the deterioration.

Summary and Conclusions

By observing the factors of the technic discussed above and summarized in the following table, it has been possible to produce uniform preparations for the histological demonstration of acid-phosphatase activity.

(1) Fixation: (a) Slices of tissue 2–3 mm. thick should be fixed in a relatively large volume of absolute acetone. This need not be chilled.
(b) Frozen-dried preparations are excellent.

(2) Dehydration: This is accomplished by the fixative. Absolute alcohol should not be used for dehydration.

(3) Clearing: Either benzene or toluene may be used. Chloroform is harmful. Tissue may be stored in the clearing agent for at least 24 hours.

(4) Embedding: Temperatures above 60°C. are harmful. The embedding should be accomplished in two hours or less. A vacuum oven shortens the time required.

(5) Mounting: The usual water bath method is not harmful provided the temperature of the water is not sufficient to melt the paraffin ribbon. Albumin or celloidin must not be used to affix the ribbon to the glass slide. No adhesive agent is required, the ribbon merely being dried on the slide for 12 to 72 hours at room temperature.

(6) Deparaffinization: This should not be done before incubation in the substrate as this radically changes the localization as well as diminishing the enzyme activity. It is accomplished incidently in clearing prior to mounting with synthetic resin.

(7) Incubation: 45°C. or below is a safe temperature.

(8) Storage of material: Uncut blocks may be stored. Cut blocks deteriorate rapidly.

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THE SERIAL SECTIONING OF DRIED MATURE CEREAL SEEDS[1]

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Received for publication, Oct. 31, 1950

ABSTRACT.—**Good serial sections of dried, mature cereal seeds can be made by first soaking the seeds for 24–48 hours in 5% aqueous hydrofluoric acid, fixing them in FAA (formaldehyde, ethyl alcohol and acetic acid solution), dehydrating in normal butyl alcohol, embedding them in paraffin, and soaking them in water for 12 hours just prior to sectioning. With this technic, one can obtain serial sections of an entire seed in one paraffin ribbon, 20μ thick.**

INTRODUCTION

Technicians have long recognized the difficulty of obtaining satisfactory serial sections of dried mature seeds without resorting to the somewhat laborious and complicated celloidin method. A simple technic for obtaining high quality serial sections of mature seeds embedded in paraffin should be of some value. The technic described below was devised primarily for the study of the structure of wheat and corn seeds, and of fungi present within these seeds, but it should be applicable to other seeds of similar structure.

The seeds used were those of maize and wheat. The wheat seeds had been stored dry for two to six years. The schedule finally determined as most satisfactory is as follows:

1. Soak in 5% aqueous hydrofluoric acid for 24–48 hours, depending on the size of the seeds being used.[3]
2. Wash in running water for 24 hours.
3. Soak in FAA (formaldehyde, ethyl alcohol and glacial acetic acid solution) for 24–48 hours, again depending on the size of the seeds.
4. Soak in two changes of 50% ethyl alcohol for at least 6 hours in each change.
5. Dehydrate in the Sass (1940) series[4] of normal butyl alcohol in ethyl alcohol beginning at approximately 40% alcohol content

[1]Paper no. 2592, Scientific Journal Series, Minnesota Agricultural Experiment Sta.

[2]Research Asst., Dept. of Plant Pathology and Agri. Botany, Univ. of Minn., St. Paul 1, Minn.

[3]Extreme caution must be used in handling the hydrofluoric acid. All work should be done under a chemical hood, and all implements should be coated with paraffin, especially the glass vials in which the material will be stored during the acid soaking period.

[4]SASS, JOHN E. 1940. Elements of botanical microtechnique. McGraw-Hill Book Co., New York and London.

(Sass' grade number 2) allowing 6 hours between each change to 80% alcohol content (grade number 5), 12 hours in each change of the higher alcohols, and 12–24 hours in each of two changes of absolute normal butyl alcohol.

6. Infiltrate with soft paraffin, allowing a minimum of 10 days in the paraffin oven to obtain satisfactory penetration. The paraffin in the embedding vials should be changed at least once in every 24 hours. Either medium or high melting point paraffin will yield satisfactory results, although best results in warm weather are obtained when the material is embedded in a high melting point paraffin. Parawax has been found entirely adequate except in very hot weather when a melting point of at least 54–56°C. was required.
7. Embed in paraffin.

FIG. 1. — Longitudinal section of entire wheat seed. The cracks in the endosperm occur when the dried seeds are hydrated.

8. Ten to 16 hours prior to sectioning, trim the embedded seeds into paraffin blocks suitable for mounting on the microtome blocks. Expose a small portion of the seed on the same plane on which the seed is to be cut. Place seeds in corked containers of clean water and put into the refrigerator away from freezing coils, or in a cool place in the room. Remove each piece from the water just prior to mounting and sectioning. Do not allow the pieces to dry out following the water soaking before sectioning.
9. Section seeds, beginning at exposed portion. The first few sections should be discarded. Best sectioning results have been obtained with a rotary microtome, using a Shick injector blade mounted in a razor blade holder. One blade is used for two seeds and then discarded. Sections can be cut from 15μ up in thickness although 20μ has been found most satisfactory. In warm weather it was found necessary to cool the blade and mounted paraffin block in ice water for a few minutes just prior to sectioning.

10. Mount sections on clean slides using Mayer's affixative.
11. Place on warming plate set at 45–50°C. for 24 hours.
12. Stain as follows: Remove paraffin in xylene. Remove xylene in 95% ethyl alcohol. Stain in alcoholic safranin for 1 hour. Rinse in water for a few seconds. Remove excess safranin with a few drops of acidified water. Rinse in water for a few seconds. Partially dehydrate with a few drops of 95% ethyl alcohol. Counterstain for about one minute with picro-aniline-blue in alcohol. (This stain is prepared as follows: Prepare saturated solutions of picric acid and aniline blue in 95% ethyl alcohol; add the picric acid solution to the aniline blue solution a few drops at a time until a medium green solution is obtained; filter before using.) Dehydrate in 95% and absolute ethyl alcohol. Clear in clove oil. Clear in xylene. Add balsam and cover slip.

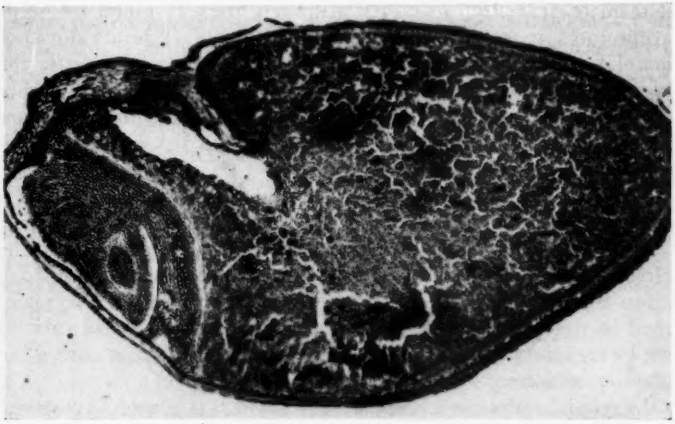

FIG. 2. — Enlarged view of a wheat embryo.

DISCUSSION

The foregoing schedule was chosen as a result of fairly extensive testing of various killing and fixing fluids, dehydrating agents, acid soaking times, and staining reactions. Of the killing and fixing fluids used, it was found that FAA and Allen-Bouin's type II (Sass, 1940) gave the most satisfactory results for sectioning and staining, but FAA is favored for general work because of the difficulty in removing the Bouin's from such bulky and dense material. Attempts to section seeds from which all the Bouin's had not been removed resulted in failure, due to the shredding of the endosperm. For careful cytological work, however, Allen-Bouin's type II seems to give better results. If Bouin's is used, the seeds must be washed in running water for at least 24 hours after being removed from the killing and fixing fluid, and dehydration must then be started at the beginning of the normal butyl series. Elimination of the killing and fixing fluid seemed to decrease the sharp color distinctions in the staining reaction.

A fairly slow dehydration in normal butyl was found to be better than rapid dehydration in normal butyl or dehydration utilizing the tertiary butyl alcohol or iso-amyl alcohol series.[5] The seeds remain soft throughout the dehydration period until placed in the higher alcohols (around 80%) when they again become hard, thus necessitating the soaking in water just prior to sectioning. Variations of the time the seed was soaked in acid, and various combinations of killing and fixing fluids and dehydrating agents have been tried in an attempt to eliminate the water soaking, but so far these attempts have been unsuccessful.

The length of the pre-soaking period in the hydrofluoric acid does not seem critical after 24 hours, although after 72 hours the seeds become too soft to handle easily. Soaking up to 72 hours does not appreciably change the staining reaction, nor does it decrease the subsequent hardening when the material is passed into the higher alcohols. Attempts to shorten the dehydration time in the higher alcohols resulted in improper dehydration and subsequent incomplete infiltration with the paraffin.

The time required for complete paraffin infiltration can be shortened by one or two days if the material is aspirated under a vacuum of 15–25 inches of mercury for a few minutes each day while in the melted paraffin. A Cenco vacuum pump and small water bath have proven satisfactory for this purpose. The water bath should be maintained at the same temperature as the paraffin oven, and caution must be exercised to prevent the paraffin from solidifying during the aspiration process.

Of various stains tried, the one described was found to give the best color contrast and structure differentiation. The pericarp and remains of the nucellus are stained a deep red, the aleurone layer blue-green, starch grains pink except for the hilum which is a deep red, almost black, and the germ tissue yellow-green (Fig. 1 and 2). In addition to clearly differentiating the structures of the seeds, this stain has been found exceptionally useful in revealing the presence of fungus mycelium within the seeds. With this stain the mycelium is stained a definite blue and cross-walls are clearly visible. The presence of even a few hyphae within the pericarp or in the germ can be detected.

This method has proven entirely satisfactory for both dent corn and wheat seeds but is not applicable to flint corn, unless the flint portion of the seed is removed before embedding. In a few cases it has been necessary to soak the larger seeds in water a second time after the seeds had been sectioned half-way through. Usually, however, this is not necessary.

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[5]This technic has been developed by Dr. Wm. S. Steil, Marquette Univ., Milwaukee, Wis., and is as yet unpublished.

A DEVICE FOR THE RAPID FIXATION OF FRESH FROZEN TISSUE SECTIONS

F. B. ADAMSTONE, *University of Illinois, Urbana, Ill.*

Received for publication Oct. 23, 1950

ABSTRACT.—**The preparation of slides from fresh frozen tissue sections requires rapid immersion in the fixative. This is necessary since the method is particularly useful in demonstrating the presence of labile enzyme systems and for the localization of various soluble salts both of which usually require rapid immersion of the section in one of the chemical reagents used in the tests. Furthermore, unless the section is immediately immersed in the fixative just as melting occurs the cells of the tissue will be completely destroyed.**

In the device described, the slide is held in a chute above a jar of fixative, and, as soon as the frozen section is placed upon it, the slide is released by an electro-magnetic device and shot rapidly into the fixative by a spring-actuated mechanism. It is obvious that this method offers a means whereby tissues may be subjected to various chemicals without any intervening exposure to fixatives, etc. Thus tests may be applied to the tissues for the detection of various substances such as nucleic acid or for the localization of the sites of activity of highly labile enzyme substances.

The success of the technic of making permanent preparations from frozen sections of fresh tissues depends to a large extent upon the speed with which the section can be immersed in the fixing fluid or other chemical reagent just at the moment when it is placed upon the clean microscope slide[1]. The reasons for this are two-fold. In the first place, the method is particularly well adapted to the study of labile enzyme systems, and for the localization of various soluble salts, etc., but the success of such histochemical tests often requires rapid immersion of the section in one of the chemical reagents used in the particular test. In the second place, immersion of the section without delay is absolutely essential to prevent injury to the cells, for, if the section is allowed to melt fully the cells of the tissue will be completely destroyed at the time of immersion. Hence, considerable speed is necessary if undistorted preparations are to be secured. This step is rendered very difficult or almost impossible if the worker has no assistance, because of the fact that both hands are occupied in manipulating the frozen section. As a result, delay is almost inevitable under ordinary circumstances but must be avoided if first class preparations are to be obtained. To overcome this difficulty the

[1] ADAMSTONE, F. B., and TAYLOR, A. B. 1948. The rapid preparation of frozen tissue sections. Stain Techn., **23**, 109–16.

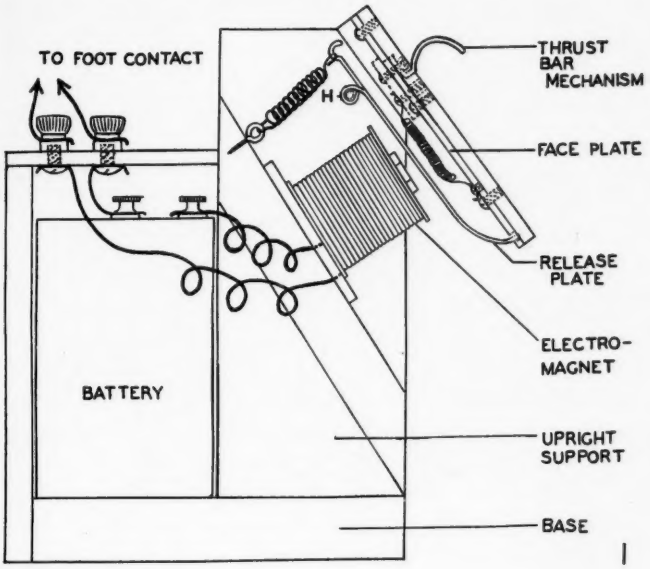
writer constructed the device described below for mechanically immersing the slide. It has proven quite successful for the purpose and hence is described for the benefit of other workers who may be interested.

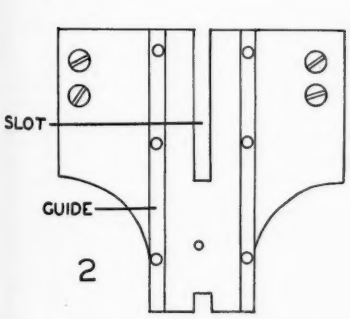
In essence the device consists of a small metal chute in which the slide is held close above the jar of fixative until the moment when the section is placed upon it. As described in a previous article (l.c.) the section is handled by means of a special section lifter in the form of a double-bottomed scoop filled with dry ice so that it remains frozen until the very moment when it is transferred to the microscope slide. A very fine camel's hair brush is used for this purpose and at the moment the section touches it the slide is released from the chute by a small catch at the lower end of a metal release plate. The release plate is operated by an electro-magnet connected with a foot switch. At the moment the slide is released, a spring-operated bar working in the slot at the upper end of the chute goes into action giving the slide a downward thrust and a speedy start down the chute. It is thus shot into the jar of fixative with considerable force, and it is well to have a rubber pad in the bottom of the vessel to prevent breaking the slide.

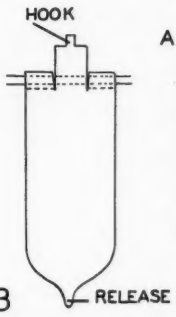
After each slide is immersed in the fixative it is transferred to a larger vessel containing the same fixative and the chute is loaded with a fresh slide ready to receive the next section. Details of the construction and parts of the immersing apparatus are outlined below and are illustrated in the accompanying figures. In Fig. 1, the general construction of the device is shown in side view. It consists of a small rectangular box to hold the batteries, with the slide chute at one end. The face plate of the chute is fastened by screws to wooden uprights 7″ x 2 ¾″ x ¾″ at the sides. The slide release plate is located beneath the face plate and is hinged at its upper end (H). Its lower end bears a small upturned catch which is held firmly in a small notch in the end of the face plate by the leverage of the spring attached at its upper end. The electro-magnet operating the release plate is fastened to an inclined wooden base running parallel to the face plate and located between the upright supports. One or more batteries is contained in the box built around the uprights. These are connected to the coil of the magnet and to the foot switch.

In Fig. 2 the construction of the brass face plate and chute is shown in face view. The general relation of the "thrust bar" to the face plate is shown as well as the spring which operates it, in Fig. 1. The face plate (4″ x 4″) is made of $^1/_{16}$″ brass. It has two parallel guides to direct the slide into the fixative. These are made of ⅛″ brass bars placed $1^1/_{16}$″ apart. The slot 2″ x $^3/_{16}$″ in the upper half of the face plate between the guides serves as a track for the thrust bar mechanism.

In Fig. 3 the construction of the release plate is shown in face







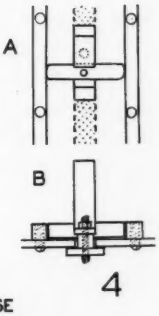
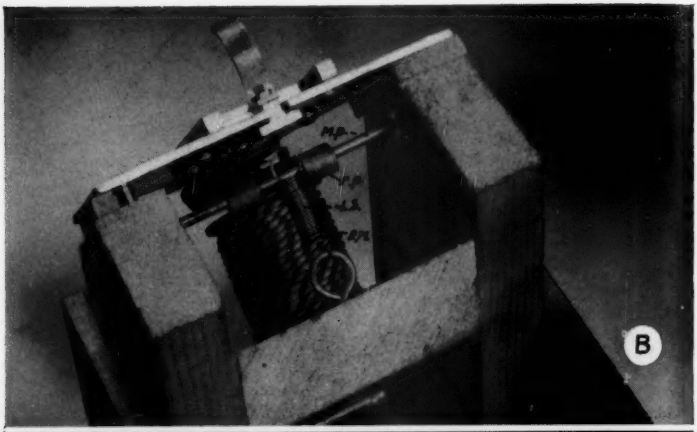


FIG. 1. Diagram, lateral view, of working parts of slide immersing device.
FIG. 2. Diagram, face view, chute in which slide is held ready to receive frozen section.
FIG. 3. Metal release plate which is located beneath chute as in figure 1.
FIG. 4. "Thrust bar" mechanism by which the microscope slide is shot down the chute into fixative. A. face view, B. end view. Side view is shown in Fig. 1.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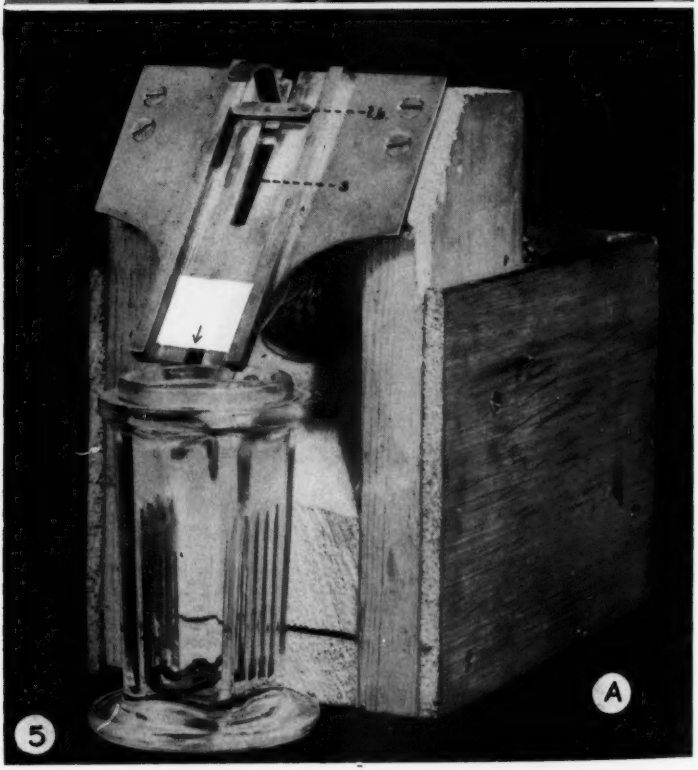
A
5

view. It is hinged at H (Fig. 1) and is held firmly against the face plate by the spring at its upper end. It is made of a strip of sheet iron $^1/_{16}$" x $1^1/_4$" and is actuated by the electro-magnet and foot switch.

Fig. 4 (a,b) shows face and end views of the "thrust" bar mechanism. Its relation to the face plate and chute is shown in Fig. 1. The "thrust bar" is attached to a metal slider which is able to move up and down freely in the slot in the face plate. The "thrust bar" is also able to move up and down freely in the space between the guides of the chute. It is really a cross bar which contacts the upper end of the slide. The slider is connected below to a lightly wound spring which is attached at its lower end by a screw beneath the face plate. This spring gives the slider a quick downward pull when the catch holding the microscope slide is released. A curved handle fitted to the upper end of the slider facilitates loading fresh microscope slides into the chute.

The device works quite successfully after a little practice has been obtained with it and is quite essential if one wishes to work alone. It greatly shortens the time interval needed for immersion of the slide in the fixative so that very rapid fixation is possible and fine cytological preparations can be made. Moreover, in experimental work involving the use of chemical methods for the detection of various substances present in the cell, e.g. nucleic acid by the Feulgen reaction, or, for the localization of the sites of activity of highly labile enzymes, it is often possible to make direct tests without subjecting the tissues to various intermediate steps which may detract from the intensity of the reaction. Many other possible uses for the method suggest themselves such as the localization of radioactive tracers. Indeed the method has already been used quite successfully by colleagues for this purpose.

FIG. 5A. Front view of slide immersing device set up with coplin jar as slide receptacle. A microscope slide is loaded in the chute ready to receive the frozen section which is placed on the lower end (shown white) so that it will hit the fixative quickly. The slide is held in the chute by the catch of the release plate just below the arrow. The "thrust bar" (t.b.) is in contact with the upper end of the slide. The spring (s.) which activates the thrust bar can be seen through the slot in the chute of the face plate.

FIG. 5B. View of release mechanism from above. The release plate (r.p.) is hinged on the metal pin (m.p.). The catch on its lower end (arrow Fig. 5A) is held against the face plate by the pull of the large spring (l.s.) hooked over its upper free end. The electro-magnet (e.m.) releases the slide by pulling the release plate down. When it does so the second smaller spring connected beneath the face plate to the thrust bar pulls the bar downward quickly thus shooting the slide into the fixative.

SIMPLIFICATION OF THE MANUFACTURE OF SCHIFF REAGENT FOR USE IN HISTOCHEMICAL PROCEDURES

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ABSTRACT.—**Schiff reagent may be made by simply adding 1 g. fuchsin, 1.9 g. sodium metabisulfite to 100 ml. 0.15 *N* hydrochloric acid, shaking at intervals or mechanically for 2 hours, decolorizing with charcoal and filtering.**

To obtain perfectly colorless Schiff reagent, it is necessary that fresh activated charcoal be used. If the final product is not water white, a fresh sample of activated charcoal should be obtained, and the product retreated and refiltered.

Schiff reagent precipitates a white crystalline substance after varying periods of cold storage, apparently a polymer which can be partly depolymerized by boiling and reconstituted into a Schiff reagent. The reconstituted reagent rather promptly precipitates again. Hence reconstitution is impractical and preparation of fresh batches at intervals of 2–3 weeks is recommended.

For some years we have been following a technic for the preparation of Schiff reagent based on the directions of Coleman (1938), de Tomasi (1936), of Bauer (1933), and of Stowell (1945). This technic has given quite satisfactory results, and the solution may be used for some weeks if kept stoppered at 5°C.

This technic required dissolving 1 g. fuchsin in 100 ml. boiling hot water, filtration at 60–80°C., addition of 2 g. sodium bisulfite or metabisulfite and 20 ml. normal hydrochloric acid, standing overnight to decolorize and filtration after shaking for 1 minute with powdered charcoal. The procedures cited in the recent textbooks[1] are essentially identical with the foregoing.

Recently in reviewing the preparation of Schiff reagent, it was observed that lots made in two laboratories from the same fuchsin differed radically in color, one lot being a rather deep yellow, the

[1]CONN, H. J., and DARROW, M. A. 1949, 1948. Staining Procedures (Pages IA–3, ID–3). Biotech. Publ., Geneva, N. Y.

COWDRY, E. V. 1948. Laboratory Technique in Biology and Medicine, *2nd ed.*, Williams and Wilkins Co., Baltimore.

GLICK, D. 1949. Techniques of Histo- and Cytochemistry. Interscience Publ., N. Y. and London.

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other water white. The difference was found to be due to the age of the activated charcoal used at the final decolorization. A sample of "activated" charcoal of some years' age had been used over a period of years with very gradual deepening of color of the final product, which continued quite satisfactory for Feulgen and periodic acid methods. A fairly recent sample, about a year old, gave the expected pale yellow, and a sample obtained from the chemistry laboratory, where considerable quantities are used continually and the available supply was quite fresh, yielded water white Schiff reagent.

In the course of these tests the attempt was made to shortcut the preparation of Schiff reagent, with surprisingly satisfactory results. It was found that the fuchsin and bisulfite could be simply weighed into a suitable bottle, the diluted hydrochloric acid added and the mixture shaken a few times in the course of 2 or 3 hours. At the end of this time a rather deep yellowish brown solution with no residue resulted. Addition of fresh activated charcoal, shaking for 1 to 2 minutes and filtration yielded a water white solution which was ready for immediate use.

Reduction in the amount of hydrochloric acid added resulted in a creamy pinkish white suspension, which cleared to the usual yellow brown solution abruptly as the concentrated hydrochloric acid was added drop by drop with shaking. The total amount of hydrochloric acid required to yield a clear solution is related to the amount of fuchsin used. For 1 g. fuchsin in a final volume of 100 ml. of 0.1 *M* $Na_2S_2O_5$, 13 ml. normal hydrochloric acid was needed, for 2 g. fuchsin 20.9 ml. was consumed. Three successive lots, 1 with 15 ml. N/1 HCl, 1 with 1 g. fuchsin and just enough hydrochloric acid to dissolve, and the 2 g. fuchsin with 20.9 ml. N/1 hydrochloric acid gave pH levels respectively of 2.2, 2.25, and 2.2.

For routine use we have settled on 1 g. fuchsin, 1.90 g. $Na_2S_2O_5$ or 2.04 g. $NaHSO_3$ (= 0.4*N* H_2SO_3), 15 ml. normal hydrochloric acid and 85 ml. distilled water. The solution is placed in a bottle with 50–60 ml. free air space above the fluid. It is shaken for 2 hours, at intervals or on a mechanical shaker, 500 mg. of fresh, activated charcoal added, shaken two minutes, and filtered on a plaited hard filter. The solution should be water white. If not, it is necessary to get fresh charcoal,[2] re-treat and refilter.

This Schiff reagent has been used with satisfactory results in the Feulgen method and in the chromic and periodic acid oxidation Schiff technics. Some lots have been kept as long as two months at 0–5°C. without evident deterioration. Several lots, using 1 or 2 g. fuchsin per 100 ml., have formed a white crystalline precipitate after varying periods of cold storage. Its presence does not appear to inter-

[2]Activated charcoal is preferably obtained directly from chemical manufacturers. A sample which on trial decolorized a yellow sulfite fuchsin solution completely, continued to do so, in practice, for about a year.

fere with the efficiency of the supernatant Schiff reagent until the quantity becomes quite large.

This precipitate redissolves with a red color on boiling in water or in the original solution. The aqueous solution redecolorizes on addition of acid sulfite, the sulfite solution on adding acid alone, and the reconstituted solution again reacts with aldehydes to form the usual red purple color. However, such reconstituted solutions again precipitate copiously in a day or two. It appears probable that the precipitate constitutes a polymer of the fuchsin sulfinic acid complex, which can be partly depolymerized by boiling.

Reconstitution of Schiff reagent in which this precipitation has occurred, appears to be impractical. Preparation of a 2–3 weeks' supply at one time seems to be the preferable procedure.

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EMBEDDING AND STAINING AMEBOID FORMS[1]

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ABSTRACT.—**The cytochemical evaluation of experimentally treated amebae requires preparative technics which do not involve extraneous material. For such work, the following simple and rapid modifications of standard procedures allowing a high recovery have been devised: embedding amebae in bulk; embedding small numbers; preservation of whole mounts; and, staining of whole mounts.**

For embedding in bulk, the amebae are fixed, dehydrated, and cleared in a centrifuge tube. The tube is initially lined with paraffin, in order to recover the amebae which on dehydration adhere to the sides. The addition of xylene both clears, and by dissolving the paraffin, releases the adherent amebae. The cleared amebae are finally transferred from the centrifuge tube to a depression slide for embedding.

In the staining of whole mounts as well as the embedding of small numbers of amebae, the entire procedure is carried out under a dissecting microscope, the amebae remaining in the same conical depression slide throughout each of the procedures. The use of microcapillary pipettes for changing reagents further insures against loss of amebae. In order to exemplify the above procedure for staining whole mounts, a detailed description for the Feulgen technic is presented.

A simple procedure for preserving whole mounts involves fixation, dehydration and embedding on a slide. The reagents are added with a pipette, and are removed by tilting the slide. The whole mounts may be stored in this condition.

The loss of large numbers of protozoa during fixation, staining and embedding has been a major difficulty. Under experimental conditions this loss is incompatible with the complexity of the procedures. Standard procedures often require the addition of extraneous material, e.g., egg white, which might inhibit or interfere with cytochemical tests.

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[1]This investigation was supported (in part) by a research grant from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

[2]Pre-doctorate Research Fellow of The National Cancer Institute of the National Institutes of Health, Public Health Service, Federal Security Agency.

None of the procedures recommended in standard references on histological technics would satisfy the following requirements: (1) a high percentage recovery; (2) absence of extraneous material; or (3) relative simplicity of procedures. It therefore became necessary to devise methods which would satisfy these requirements in the handling, for example, of experimentally treated amebae. The technics outlined below proved satisfactory for work with amebae; and should be applicable, with only a slight modification, to other protozoa.

A. Embedding in Bulk

The difficulties encountered in embedding amebae in bulk are due to: a) the small size of the forms, b) their invisibility when cleared, and c) their tendency to adhere to surfaces when dehydrated. The loss due to adhesion has been overcome by using paraffin-lined centrifuge tubes. This provides a surface to which the amebae adhere during dehydration. The paraffin is subsequently dissolved by the clearing agent, thus releasing the amebae which may then be concentrated by centrifuging.

PROCEDURE

1. Pipette amebae (cultured after Brandwein, 1935) into a paraffin-lined centrifuge tube (15 ml. capacity) and centrifuge until the amebae have settled to the bottom. (A hand centrifuge was found to give adequate separation).

2. Remove the supernatant fluid with a pipette taking care not to redisperse the amebae. Add fixative and allow to act for 3 to 5 minutes. Effective fixatives were ethanol-formol (1 part formalin to 9 parts absolute ethanol), tertiary-butanol-formol (1 part formalin to 9 parts absolute butanol), and Carnoy's ethanol-acetic-acid mixture (1 part glacial acetic acid to 3 parts absolute ethanol).

3. Concentrate the amebae again by centrifuging, then remove the supernatant fluid. Add absolute ethanol and allow to stand 3 to 5 minutes.

4. Recentrifuge the suspension and remove the supernatant fluid. Depending on the fixatives used and completeness of removal of the fluid, steps 3 and 4 should be repeated.

5. Add xylene and allow the amebae to clear for 3 to 5 minutes, then concentrate. Chloroform proved unsatisfactory because its volatility made concentration difficult. The centrifugation time required for concentrating the cleared amebae is determined by trial, using eosin-stained specimens.

6. Carefully transfer the amebae, from the bottom of the xylene-filled centrifuge tube into melted paraffin at 58°C. A small round depression slide[3] (16 mm. diameter × 3 mm. depth) was found most

[3]Microslides, culture (Standard Scientific Supply Co. #7396).

useful as an embedding dish. The depression was coated with glycerol prior to use.

7. Place the slide into the oven (58°C.) and leave until the amebae have sunk to the bottom of the depression and the xylene has risen to the top. Remove the supernatant fluid with a warm pipette and add fresh melted paraffin. Repeat this procedure, i.e. removal and addition, twice at ten-minute intervals.

8. After three changes of paraffin, solidify by immersion in ice cold water and remove the paraffin button from the depression slide.

9. Mount the top of the button (which requires no trimming) to the face of the carrying disc of the microtome. The embedded organisms are now ready for sectioning.

B. Embedding Small Numbers of Amebae

The bulk method of embedding is not satisfactory for studies involving small numbers of experimental animals. The procedure which follows was devised for handling amebae (less than 20) with little or no loss. Specimens are both fixed and embedded under the dissecting microscope (17×), in a dish having a small conical depression[4]. Fluids are removed with microcapillary pipettes (outside diameter of 0.25 to 0.5 mm.) which are prepared by drawing out the tip of a capillary pipette in a microburner.

PROCEDURE

1. Transfer the amebae to the glycerol-coated depression of the embedding dish, and pipette off most of the culture medium.

2. Add fixative, consisting of ethanol-formol (1 part formalin to 9 parts absolute ethanol), and leave 1 to 3 minutes.

3. Pipette off the fixative and wash the amebae twice with absolute alcohol, and then twice with xylene.

4. Remove the xylene and add xylene-paraffin (saturated at room temperature).

5. Place the embedding dish into the paraffin oven, 58°C., and carefully add one drop of molten paraffin to the side of the depression. After 10 to 15 minutes, carefully pipette off the paraffin which contains some xylene, and add fresh melted paraffin.

6. Continue as in the final steps under embedding in bulk.

C. Preservation of Whole Mounts

Under experimental conditions which require the periodic withdrawal of small numbers of amebae, it is essential to test (i.e. stain cytochemically, etc.) experimental groups simultaneously; especially when transitory color reactions are produced. When sections are used, this can of course be done readily. In some instances, however, it is necessary to utilize whole mounts rather than sections. In order

[4]Balance rest, glass (Standard Scientific Supply Co. #1925).

to make this possible, the procedure outlined below has been devised for embedding whole amebae on a slide.

PROCEDURE

1. Place a drop of amebae culture on a slide, allow the amebae to flatten and adhere. Holding the slide at a slight angle, drop the fixative (see under embedding in bulk) on the flattened amebae. Allow to stand for 1 or 2 minutes and then drain off the excess fluid.
2. Add absolute ethanol, leave for a minute and drain. Follow by the same treatment with xylene.
3. Add a drop of xylene-paraffin (saturated at 58°C.) to the cleared amebae, and place the slide on a hot plate, 50°C., for 5 to 10 minutes. On removing from the hot plate, gently rock the slide so as to obtain a uniform cooled layer of paraffin.

D. Staining of Whole Mounts

The wet smear technic for fixing amebae to slides has been recommended by many authors. In our hands its use, with numerous variations, resulted in losses ranging from 10 to 100%. Celloidin prevents such losses, but its use obscures the fine cytological details. The procedure outlined below insures a high percentage recovery. This procedure has been successfully used with modifications for iron-hematoxylin and safranin staining as well as for the Feulgen-Schiff reaction, losses ranging from 0 to 30%. Controls may be carried through, in another depression slide, at the same time, insuring similar treatment, reagents, and hydrolytic time.

The following description is for the Feulgen technic, in which the leucofuchsin (Schiff) reagent was prepared by bubbling SO_2 through a solution of basic fuchsin[5], as described by Rafalko (1946). The general procedure followed was that of Rafalko; minor modifications necessary to adapt this method to the use of the depression slide technic are indicated below.

These procedures are carried out under the dissecting microscope (17×) in square tissue culture depression slides[6] (36 mm. diameter × 5 mm. depth). Fluids are added and removed with capillary pipettes.

PROCEDURE

1. Place the amebae in the depression slide and add the fixative (1 part formalin in 9 parts 50% ethanol).
2. Dilute the fixative by the dropwise addition of distilled water, until the depression is full. Remove the fluid and repeat washing with distilled water at least five times. This amount of washing is

[5]Basic fuchsin, dye content 91%, certification No. NF54, a product of the National Aniline and Chemical Co., Inc.

[6]Microculture slides (Arthur H. Thomas and Co. #7048–c).

necessary in order to remove any trace of formalin, which itself would produce a positive Feulgen reaction.

3. Remove a major portion of the final wash water and after covering with a glass plate, place the slide in a hot air incubator at 58°C. for 15 minutes (for temperature equilibration).

4. Add 1*N* HCl, previously warmed to 58°C. and allow hydrolysis to continue for 10 to 13 minutes. On removal from the incubator, add cold distilled water to interrupt hydrolysis.

5. Wash the amebae two or three times with distilled water, add 5 to 7 drops of Feulgen-Schiff reagent and allow to stand for 1 to 1½ hours.

6. Remove the Feulgen reagent, and wash the amebae three times with sulfurous acid (water saturated at room temperature with SO_2 gas). Allow at least 10 minutes for each washing.

7. Remove the amebae from the final wash and transfer to a clean cover slip.

8. Fix the amebae to the cover slip by the addition of 95% ethanol; rinse several times with 95% ethanol, and mount in Euparol (Grübler) or Diaphane (Will Corp.).

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THE ACETO-CARMINE TECHNIC APPLIED TO THE COLONIAL VOLVOCALES

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A study of nuclear structure and chromosome number in the colonial Volvocales has led to the development of an aceto-carmine technic particularly applicable to this group of plants. The genera investigated are *Gonium, Volvulina, Eudorina, Pleodorina,* and *Volvox*. Another genus, as yet undescribed in the literature, has also been investigated successfully by this method.

FIXATION

The fixative used is a variation of Carnoy's acetic alcohol, consisting of 3 parts absolute alcohol and 1 part glacial acetic acid saturated with iron acetate (Cave and Rattenbury, unpublished). Material from cultures (Juller, 1937; Pringsheim, 1946) or from field collections is fixed (1) directly on the cover slip or (2) in small vials.

(1) When the colonies are to be put on the cover slip, they are first picked out by a capillary pipette under the low power of the microscope and transferred to the center of a cover slip. If material is scarce or difficult to handle a drop of concentrated iodine in potassium iodide may be added to the water under the microscope to kill the colonies first. Use of iodine enhances nucleolar staining. The presence of many other organisms may sometimes make it necessary to transfer the contents of the pipette to another drop of water and to pick out the species in question a second time. After sufficient numbers have been accumulated on the cover slip a drop of the fixative is placed directly on the material, and the cover slip is put on a piece of glass or flat cork inside a small petri dish. A few drops of fixative are added in the dish to retard evaporation. Colonies may be kept in this way for several days if they are examined periodically to see that they do not dry out. However, the material can be stained even 15 minutes after fixing, although fixation for several hours seems to be better.

(2) If large amounts of good material are at hand or when no time for immediate staining and observation is available, the colonies may be stored in vials. They are picked out as above but instead of being put on cover slips they may be transferred to small vials of fixative. A variation of this method is to pipette living colonies into vials of water containing a drop of iodine in potassium iodide. They may be allowed to stand in this any time from 5 minutes to 2 hours. After the colonies have settled, the supernatant fluid is carefully removed by a pipette and replaced by the fixative. Material kept in the fixative for over a year has been found to be still in excellent

[1]Work done while holding a Senior Bursary from the Council for Scientific and Industrial Research of the Union of South Africa.

condition. In making preparations from material in the vials a small amount is taken up in a pipette from the bottom of the vial where the colonies have settled, and transferred to the center of a cover slip.

Staining

The aceto-carmine is made up by refluxing 2 grams of National Aniline carmine in 400 ml. 45% glacial acetic acid for four hours. The solution is cooled before filtering.

Before staining, the fixative on the cover slip is drained off and allowed to evaporate to a point just short of letting the colonies become dry. The inverted cover slip is then placed on a drop of aceto-carmine on a slide. After several minutes the slide is heated over an alcohol lamp or small gas burner to just below the boiling point. If there are a few bubbles on the slide it is easy to remove it from the flame when they begin to expand and thus prevent boiling. In studying chromosome number it usually will be found advantageous to squash the colonies in varying degrees according to the species. This has to be determined by trial and error for the species under consideration. If squashing is needed, a small drop of 45% acetic acid is placed at the edge of the cover slip and the slide reheated. Then, before cooling, it is squashed gently or severely as the case may be. If no iodine has been used the chromosomes from prometaphase to early telophase are black and the nucleolus a gray red. If iodine has been used, the nucleolus is more heavily stained and the chloroplasts and cell membranes are more distinct. Under some conditions, even for the same species, addition of iron to the stain helps to stain the chromatin, although, as a rule, the iron in the fixative is sufficient.

Permanent Preparations

Slides may be made permanent in euparal or diaphane by first adding the following solutions to one side of the cover slip and drawing them off with filter paper from the other side: (1) 45% glacial acetic acid; (2) one part 95% alcohol to two parts 45% glacial acetic acid; (3) two parts absolute alcohol to one part glacial acetic acid; (4) absolute alcohol; (5) absolute alcohol. To save time several slides may be treated at one time with the first solution and left in a large covered petri dish for 10 minutes to several hours, then blotted and treated likewise with the other solutions.

The cover slips are next ringed with euparal or diaphane and placed in a flat, tightly covered tin box (Bradley, 1948), the bottom of which is covered by filter paper wetted with a few drops of absolute alcohol. After 24 hours they are put on top of the paraffin oven to dry overnight. Cover slips may then be cleaned with absolute alcohol without danger of their slipping.

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THE RECOGNITION OF LIPOLYTIC ACTIVITY WITH THE MICROSCOPE

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In view of its probable wide applicability, it seems desirable to publish a note on a simple technic for the recognition with the microscope of the action of lipase. In brief, the method is to make an emulsion of neutral fat previously stained with a red Sudan stain, subject some of the emulsion to the action of the supposed lipase for an appropriate time and then examine with the microscope a recovered drop of the emulsion in a solution of Nile blue sulfate. It has long been known that Nile blue sulfate stains liquid neutral fats a reddish color and fatty acids blue.

The procedure as used by the author in a special problem is as follows: 60 ml. peanut oil previously saturated with Sudan IV[1], 15 g. powdered acacia and 30 ml. distilled water were put in a Waring blendor and thoroughly mixed. The resulting product was thinned somewhat by the gradual addition in the blendor of 30 ml. additional water. This procedure gives a satisfactory stock emulsion with particles ranging in size from less than one μ to perhaps ten μ. Oil immersion examination of a diluted drop of the emulsion shows the larger droplets to have a clear pink color; a slight but definite pink is detectable in the medium sized droplets. Definite color was not recognizable in the smallest individual droplets. Since Sudan IV stays with the fatty acid radicle, one is able subsequently in experimental material to identify the introduced droplets, except the very small ones, whether they be free or intracellular and whether they be in the form of neutral fat or fatty acid. Even the very small droplets when present in large numbers will give a diffuse pink color to sections of epithelium, for example.

With a pipette drops of the emulsion were inserted into the stomachs of oysters and other bivalves or fed to them in suspension in agitated sea water. After two plus hours a drop of the stomach contents was withdrawn through the mouth with a pipette and examined with the microscope. An abundance of pink stained droplets was observed. Most of these were free but some were present in leucocytes, which appear in the stomach in large numbers. A drop of Nile blue sulfate solution (Grübler's stain used) was placed at the edge of the cover glass. As the dye diffused under the cover glass some of the droplets changed from a pink to a definite blue, the number

[1]Author's sample labeled "Scharlach R".

of blue droplets and to some extent the intensity of the blue varying with the length of exposure of the fat to the stomach contents. With material from animals that had been fed at intervals over a period of days with Sudan IV stained emulsion in sea water, all of the droplets and blobs of oil were pink before Nile blue and virtually all were a brilliant blue after Nile blue. The concentration of the Nile blue stain does not seem to be particularly significant. A solution of 1:10,000 was very satisfactory.

As controls, samples of the stock emulsion were tested. No droplets turned blue; in fact the larger ones showed a deeper orange pink when the action of the Nile blue sulfate on neutral fat was superimposed on the pink of Sudan IV. Also a drop of the stock emulsion was mixed with an aqueous solution of lipase (steapsin obtained from Nutritional Biochemicals Corporation) and subsequently treated with Nile blue sulfate solution. The color response was the same as that observed following subjection of fat emulsion to the action of stomach juices of the oyster:—there was an increasing number of blue granules and an increasing intensity of blueness with the passage of time.

THE EXAMINATION OF TISSUES IN LIVING LEAVES AND FLOWERS BY MEANS OF A HIGH VACUUM TECHNIC[1]

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ABSTRACT.—**The cells of living leaves and flowers can be prepared for examination in toto by replacing the intercellular air with physiological salt solution. This is done by mounting all or parts of leaves and flowers in salt solution and subjecting the preparation to a vacuum of close to 0.1 μ of mercury. The solution is allowed to boil a few moments, the vacuum is slowly released; then the tissues are ready for examination. Preparations in many ways far superior to chemically cleared mounts can be made in one minute.**

One can see very little structure in leaves and flowers that contain air in the intercellular spaces if they are merely mounted in water and examined with a microscope. When illuminated from below they are not sufficiently clear for microscopic vision because light is scattered within them by the moist surfaces of cells that glisten in the air spaces. It was presumed that were it possible to completely replace the intercellular air with physiological salt solution, leaves and flowers would be rendered sufficiently transparent for observation of both epidermal and inner cells in an undisturbed living state. This can be accomplished if the air is exhausted *with a mechanical high vacuum pump capable of boiling water in a few seconds at room temperature.* Excellent preparations can be made with a pump rated at 0.1 μ Hg vacuum with a free air capacity of 21 liters per minute. The pump is closely connected to a small bell jar equipped with a release valve. The bell jar, placed on a vacuum plate, is convenient for processing portions of leaves and flowers mounted on slides.

Small leaves and entire petals of small flowers can be processed advantageously. Pieces of leaves or flowers one square cm. in area can be processed but portions 2 × 4 cm. or larger, are more satisfactory, especially if 2 × 3 inch slides and 48 × 60 mm. cover glasses are available to make the preparation. It seems that the larger the subject from which the air is removed, the less the danger of the air re-entering the tissue when the vacuum is released. Also, to prevent air from re-entering the tissue after the high vacuum has been released, it is

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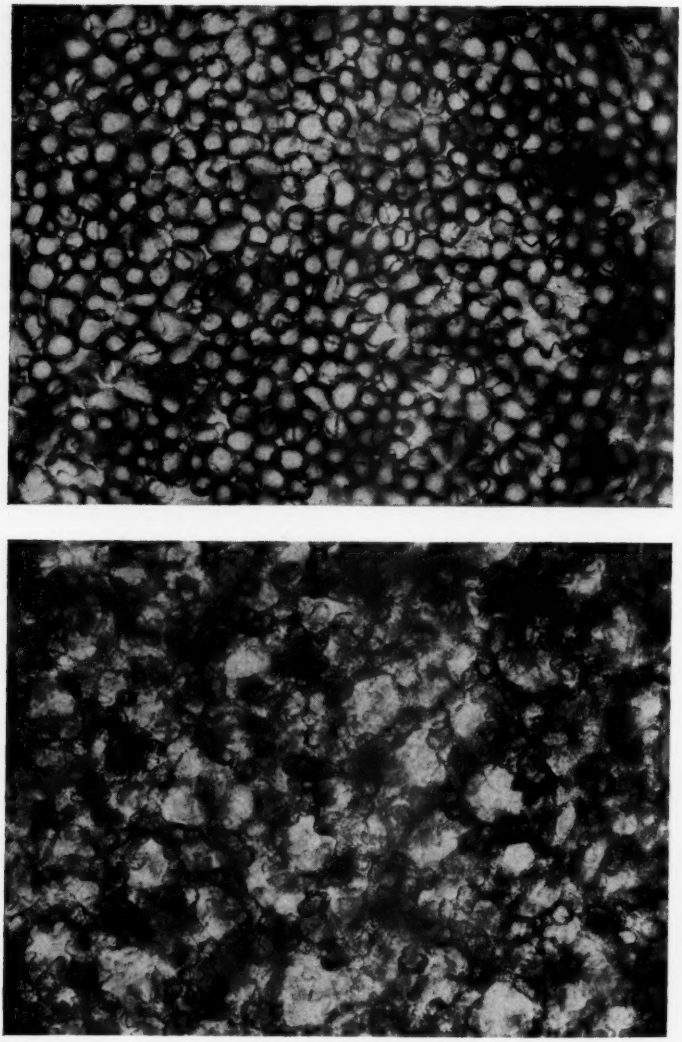

PLATE 1

Looking into a living leaf of tobacco by means of the high vacuum technic. Top: View through the upper epidermis into the palisade cells. Bottom: View through the lower epidermis into the spongy chlorenchyma cells. Both photographs made at 100× with 16 mm. coated apochromatic lens. Filter F.; film, Panoatimic ×; full development in DK 50. Illuminated with 16 mm. condenser cone.

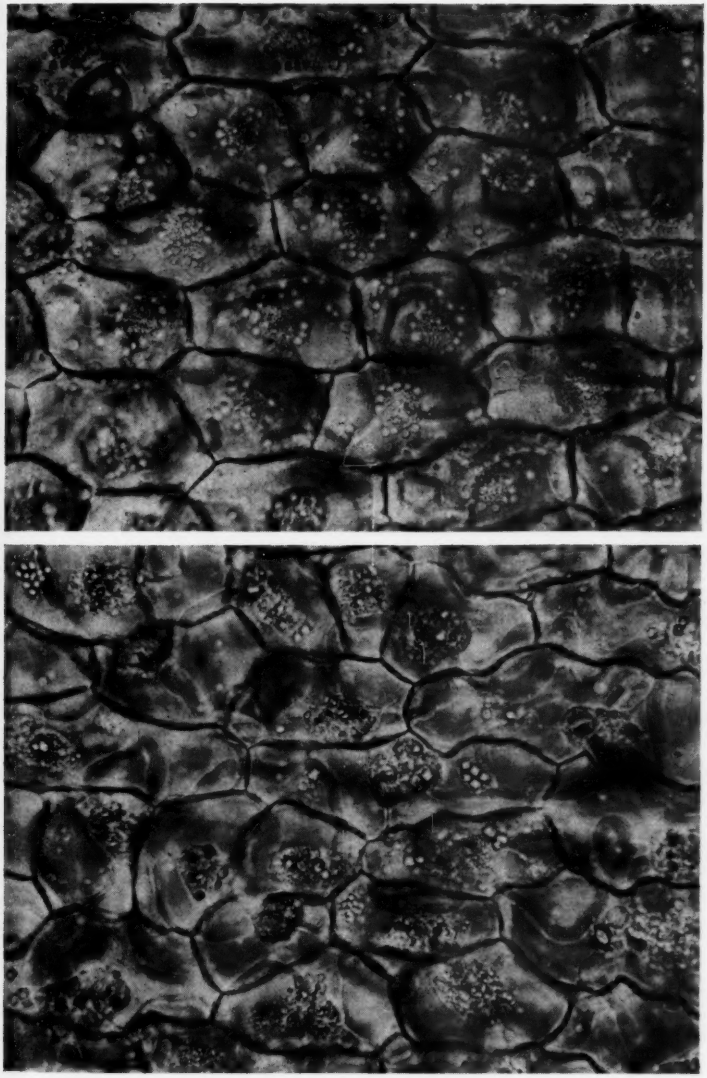

PLATE 2

Top: Epidermis of *Vicia faba* flower photographed on living standard (petal) at 500 ×. This is a healthy flower. Note the leucoplasts clustered around the nuclei. Bottom: Epidermis of *Vicia faba* showing the visible effects of Bean Virus 2 in the standard of the living flower. Note the complete rearrangement of cytoplasm, nuclei concealed in viroplast masses. The shiny crystals are indications of the virus. The formation of the virus entities in living flower can be observed by the vacuum technic.

Both photographed at 250 ×, printed at 500 ×. Lens, 8 mm. coated apochromat; Filters H – 56. Film. Contrast Process Ortho; very short development in DK 50. Illuminated with 16 mm. condenser cone.

advisable to use a cover glass at least twice the size of the subject. If a standard slide is used, covers 22 × 40 mm. or larger are necessary to cover the material adequately.

The subject should be flooded with physiological salt solution so that the liquid extends beyond the cover glass and then treated in the following manner: Exhaust the preparation until the solution "boils" vigorously for about five seconds at room temperature; stop the exhausting and then slowly release the vacuum; remove the slide and gently tap the cover. One such treatment is usually sufficient. If dark lines denoting air pockets are apparent, replace the slide and exhaust again to the same end point. The subject should appear translucent throughout when ready for examination.

For observations within living leaves and flowers, objectives of the 22.7 mm., 0.17 n.a. group are ideal for general histology; the 8 mm. apochromats are suitable for observing plastid variations in leaves and virus entities in flowers; and lenses of the 4 mm., 1.00 n.a. and 3 mm., 1.15 n.a. groups are necessary for observing and photographing nuclear structures in cells within leaves and flowers.

This simple procedure should have many uses. We employ it to observe the histology of virus symptoms such as "chlorosis", "vein clearing", and "necrotic rings". The behavior of cell inclusions from virus infection can be watched in living tissues under controlled conditions for as much as eight hours. Especially beautiful preparations can be made of infected living flowers of leguminous plants to demonstrate virus entities. The progress of fungus infection can be followed. Preparations of living leaves enable students to visualize the structure of spongy parenchyma that by other methods is difficult to see.

It is probable that had high vacuum equipment been generally available to plant histologists, this technic would have come into general use long ago.

TEMPORARY MOUNTS FOR POLLEN ABORTION DETERMINATIONS[1]

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Abstract.—**Two methods of preparing temporary maize pollen mounts are described. These have been designated as the "agar" method and the "Karo" method, respectively. It is possible with either method to obtain reasonably homogeneous mixtures of normal and aborted pollen grains evenly distributed on microscope slides.**

In both methods the pollen is prestained, while still in the anther, in a concentrated solution of I_2KI. The anther is then transferred to a drop of the medium on a slide and dissected. Following forcing out of the pollen grains and removal of anther debris, the drop is stirred to effectively distribute the two types of pollen grains.

In the agar method, a small drop of a hot 1% aqueous agar suspension is placed on a previously-warmed slide. The drop is spread into a film and stirring is continued until the medium has become sufficiently firm to retain the pollen grains in place. A slight pressure on the cover glass results in a uniformly thin film.

Colorless Karo, either pure or slightly diluted with water, is the mounting medium in the second method. The drop of medium should be of such size that when the cover slip is applied the medium (and not the larger pollen grains) will support the cover slip. Following stirring, the cover slip is dropped from a horizontal position onto the drop. The completed slide must be kept in a level position until used.

Percentages of aborted and normal pollen are commonly determined by counting randomly-selected areas of relatively small size on mounts containing the pollen of a portion of an anther, of an entire anther, or of several anthers. Since this procedure involves sampling, a reasonably uniform distribution of the two types of pollen grains is a prerequisite to making accurate determinations.

In the past, water has been the principal mounting medium employed in making preparations for pollen abortion determinations, but it is not entirely satisfactory for this purpose. It is a well-known fact that when water is used a disproportionately large number of the aborted grains are found toward the outer edges of the preparation (Brink, 1927).

In this connection Valleau (1918) suggested the use of high vis-

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cosity liquids, "... the viscosity of which holds both the normal and aborted grains in place until the cover has finally settled firmly." In the current experiments, a satisfactory solution to the problem was developed by using media of much higher viscosity than that suggested by Valleau. Of the various media tested, a 1% aqueous agar suspension and a glucose solution (commercial crystal white Karo syrup) were found to be the most satisfactory. These two media are employed in the two methods proposed for making temporary pollen mounts designated as the *agar* and the *Karo* methods, respectively.

The Agar Method

In the agar method, the pollen grains are prestained in a concentrated I_2KI solution while still in the anther. The anther wall is punctured to facilitate entry of the reagent. Following prestaining, the anther is placed in a small drop of hot 1% aqueous agar suspension on a previously warmed slide. The drop of agar should be of such size that the area ultimately covered by the agar film is no greater than that of a single field under a dissecting microscope.

The remaining steps are carried out under a dissecting microscope equipped with a white base plate. A direct source of illumination from above is used. A combination of a 1× objective and a 9× ocular has been found to provide a satisfactory magnification.

The anther is dissected, the pollen grains are forced out of it, and the anther fragments are removed from the slide. The solution is stirred vigorously while it is still hot to spread the drop into a film and at the same time to effect a reasonably uniform distribution with respect to both number and type of pollen grains over the area covered by the agar. The stirring must be accompanied by continuous and careful inspection to make certain that the desired distribution is attained.

Stirring is terminated when the proper distribution of pollen grains has been attained and as soon as the medium has become sufficiently firm to hold the pollen grains in place. This is prior to the time of gelatinization. The preparation is allowed to stand for a short additional time so that the pollen grains will be held still more firmly. In this way they will, for the most part, retain their relative position when the cover slip is applied. A slight pressure on the cover slip results in the desired uniformly-thin film of agar under it.

Mounts to be held for more than two or three hours should be stored in a moist chamber.

The Karo Method

In using the Karo method, the medium may consist either of the pure syrup or of a mixture of one or two parts of water to 20 parts of syrup. Use of the latter facilitates getting the pollen grains more nearly into one focal plane in the completed slides. The pollen is prestained as in the agar method. Following staining, the anther is

placed in a rather large drop of medium, dissected, and the pollen grains are forced out of it.

The anther debris is removed from the slide. Even with careful handling a considerable quantity of pollen will be removed with the debris and the adhering medium. This is probably not very objectionable in most studies, as data secured by us on maize pollen indicated that the ratio of the two types of grains is not materially altered by the removal.

The material is stirred until a reasonably homogeneous mixture is obtained. Care must be exercised in this procedure to retain as completely as possible the hemispheroidal shape of the drop. Only in this way will there be a uniform movement of medium and the two types of pollen grains to the outer edges when the cover slip is applied. Some difficulty may be encountered as a result of a film forming over the surface of the Karo drop when the relative humidity in the surrounding atmosphere is low. This condition may be alleviated by diluting the Karo as suggested above and also by stirring just prior to application of the cover slip. Three-fourths inch square cover slips have been found more desirable for this purpose than those of larger size. They are dropped onto the medium from a horizontal position.

It is at this stage that the importance of having a sufficiently large drop of medium becomes apparent. When the cover slip is applied, the two types of grains are carried outwardly at the same rate as long as the film is thick enough to prevent the cover slip from falling to the level of being supported by the larger grains. Therefore, to insure a homogeneous distribution, there must be a thick enough film so that movement of none of the grains is arrested until the medium with grains has reached all edges of the cover slip. With this much medium, the cover slip is then held up by surface forces at the edges of the film and with no accompanying selective force acting on the normal pollen grains. The result is a mount with a reasonably homogeneous mixture and uniform distribution of pollen grains. On the other hand, if the amount of medium is small enough so that the cover slip comes to rest on the larger pollen grains, only the smaller grains will be spread to the outer portions of the mount with the continuing movement of the medium.

The difficulties to be encountered with the use of an excessively large drop of medium are obvious. However, it may be well to point out that, for most studies, movement of some of the medium and pollen grains therein beyond the edges of the cover slip is not objectionable. The ratios of the two types of maize pollen grains under and outside the cover slip with this type of preparation were found to be substantially the same.

Because of the nature of these mounts, the slides must be kept level prior to and during the time they are used.

Comparison of the Various Methods

Although mere inspection showed that the distribution of the two types of pollen grains was more uniform in the agar and Karo mounts than in water mounts, a statistical evaluation of the three methods was made as follows: mounts were made by the three methods from pollen samples taken from plants heterozygous for a translocation, which gave a ring-of-four at diakinesis and approximately 50% pollen abortion. All the pollen in each mount was classified on a strip-by-strip basis, each strip being 5 mm. in width.

An analysis of variance was computed and an F test made to determine if the variances obtained on mounts from the three methods of preparation differed significantly. The sources of variation were among slides and among strips within slides. In all comparisons the variances from the agar and Karo mounts were smaller than those from the water mounts. Variances from the two proposed methods were in no case significantly different from each other, and they were in all cases different from the water method at the 2% level.

One of the important considerations in the choice of a mounting medium is its refractive index. Determinations in our laboratory using an Abbe refractometer showed the refractive index of a 1% aqueous agar suspension was 1.35 at 29°C., while that of the Karo was 1.48 at 24.9°C. The refractive index of the aborted pollen grains appears to be close to that of Karo as they are much more difficult to detect in the syrup than they are in an agar solution. Detection is rendered even more difficult in Karo because aborted grains are often almost entirely collapsed, whereas in the agar medium they are usually completely expanded. Greater difficulty is encountered in getting the pollen grains into a single focal plane with Karo (even when diluted) than with agar. Probably the greatest advantage of the Karo method over the agar method lies in the comparative ease with which the desired mixture and distribution of pollen grains is obtained.

It should also be borne in mind that since there are more pollen grains per unit area in the agar mounts, two to three times as large an area must be counted on Karo mounts as on agar mounts in order to obtain pollen samples of comparable numbers.

The water method has the distinct advantages of speed and simplicity of preparation over the agar and Karo methods. It is satisfactory where only gross differences in degree of pollen abortion are to be determined.

Acknowledgement

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THE DEMONSTRATION OF COMPOUNDS CONTAINING CARBONYL GROUPS IN TISSUE SECTIONS[1]

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ABSTRACT.—**Observations have shown that the Ashbel-Seligman reagents for carbonyl compounds stain the same structures revealed by Schiff's leucofuchsin after analogous procedures. These observations suggest that carbonyl groups are the responsive configurations in the group of methods utilizing Schiff's reagents for demonstrating various lipids, carbohydrates and nucleoprotein. Although qualitatively similar results are obtained with the two procedures, the intensity of staining varies with the two methods. No explanation for this differential sensitivity is known at present.**

Leucofuchsin, prepared by decolorizing basic fuchsin with sulfurous acid, becomes recolorized upon the addition of aldehydes to its solution. This color reaction, first described by Schiff, has been used extensively in histology for detecting aldehydic compounds. It is the generator of color in the Feulgen reaction for desoxyribonucleoprotein, the carbohydrate moiety of which becomes reactive after hydrolysis in warm acid. It is frequently used for staining lipids, some of which react directly and some of which require prior oxidation by agents such as mercuric chloride. Glycogen, mucus and other polysaccharides may be stained by the Schiff reaction following oxidation by periodic acid, chromic acid, permanganate or other oxidizing agents. However, exact interpretation of the reaction is hampered by ambiguities. The Stedmans (1943a, 1943b, 1944) have suggested, and Stowell (1945) has denied, that an affinity between the recolorized leucofuchsin and certain tissue elements may cause their coloration by a simple staining process rather than as a microchemical reaction. Lison (1936) has shown that some ketones and highly unsaturated substances recolorize leucofuchsin. Dempsey and Wislocki (1946) and others have suggested, on the basis of histophysiological observations coupled with other histochemical methods, that ketosteroids react positively in the adrenal cortex, ovary, testis and placenta. This suggestion has been actively criticized (cf. Dempsey 1948). In consequence of all the claims and counterclaims, it can only be said that

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some, but not all, carbonyl compounds will recolorize leucofuchsin and that, indeed, unsaturated substances lacking carbonyl groups entirely may occasionally energize active responses.

Recently, Ashbel and Seligman (1949) have introduced a new carbonyl reagent into histology. This is the hydrazide of naphthoic acid which, like other hydrazines, energetically reacts with carbonyl groups. The hydrazide and its reaction products are colorless, but either may be revealed by coupling with diazonium salts. Thus, histochemically, the tissue is reacted with the hydrazide which is in turn coupled with and rendered visible by the diazonium salt. Ashbel and Seligman have applied this method principally to the detection of the carbonyl lipids of the adrenal cortex and other steroid-producing endocrine glands. It is apparent, however, that these reagents should also detect the carbonyl groups in glycogen, mucus, desoxyribonucleic acid and other compounds if the proper technical conditions were met. We shall show, in the following sections of this report, that this prediction is borne out.

Material and Methods

Tissues from normal rats were used. These included adrenal gland, liver, kidney, lung, peripheral nerve and the tracheal-esophageal region, representing thyroid, striated muscle, esophagus, cartilage and trachea.

For the *demonstration of lipids,* blocks were fixed in 10% buffered formalin for 24 and 48 hours, for 1 week, 2 weeks, 1 month and 6 months. Frozen sections were cut and the Ashbel-Seligman and Schiff's leucofuchsin reactions were performed. Since the Schiff method is well known, we shall here present only the Ashbel-Seligman method in detail.

Solutions:

1. *50% alcohol,* prepared from aldehyde-free absolute ethyl alcohol
2. *50% acidified alcohol*

   50% aldehyde free alcohol... 95 ml.
   Glacial acetic acid..... 5 ml.
3. *0.1% 2–hydroxy 3–naphthoic acid hydrazine*[3]

   The hydrazine is finely powdered in a mortar and dissolved in 50% acidified alcohol in a volumetric flask. The mixture is heated to 65°C. and shaken vigorously to facilitate solution.
4. *Sorenson's buffer pH 7.2 – 7.5*

   1/15 Molar primary sodium phosphate.... 28 ml.
   1/15 Molar secondary sodium phosphate.... 72 ml.

[3]Prepared by Dr. Arnold Seligman, according to the procedure described by Seligman, Friedman and Herz, 1949. These compounds are available through the Dajac Laboratories, 3430 W. Henderson Street, Chicago 18, Illinois.

Before use, an equal volume of aldehyde-free absolute alcohol is slowly added.

Procedure:

1. Wash the formalin-fixed tissue block thoroughly before sectioning. (Additional washing of the frozen section is recommended, since even traces of formalin will react with the hydrazine.)
2. Incubate in the hydrazine solution at 60°C. for 45 minutes.
3. Wash in several changes of 50% acidified alcohol.
4. Wash in several changes of 50% alcohol.
5. Wash repeatedly in distilled water.
6. Transfer to 50 ml. of alcohol-buffer solution. Stir in 50 mg. of naphthanil diazo blue B[3], dissolved in 2 drops of distilled water. (This solution is unstable and darkens quickly.) Stain for 2 minutes.
7. Wash in several changes of distilled water.
8. Mount in glycerine jelly.

Controls: To determine the solubility of the reactive materials, in the staining of lipid droplets, some sections were extracted in acetone for 1 hour at room temperature.

For the *demonstration of polysaccharides,* blocks were fixed in Rossman's fluid (picric-acid-alcohol-formalin), and paraffin sections were prepared. The deparaffinized sections were oxidized with 1% periodic acid for 5 minutes, or 4% chromic acid for 1 hour, and stained with either the Ashbel-Seligman or Schiff's leucofuchsin reagents. In order to differentiate between glycogen and mucopolysaccharides, slides were subjected to digestion in saliva prior to staining. With this treatment, glycogen is removed and the mucopolysaccharides are unaffected.

For the *demonstration of desoxypentosenucleoprotein,* blocks were fixed in Zenker's fluid and embedded in paraffin. The deparaffinized sections were hydrolysed in 1*N* HCl at 60°C. for 5, 15 and 30 minutes, and then stained with Schiff's leucofuchsin reagent (Feulgen reaction) or by the Ashbel-Seligman procedure.

Results

Lipids. In sections of rat's adrenal gland fixed in formalin for 24 hours and stained by the Ashbel-Seligman method, there was a reactive layer of cells at the periphery of the cortex, comprising the zona glomerulosa and the outer part of the zona fasciculata (Fig. 5). In these cells, the lipid droplets were intensely stained and the background cytoplasm was completely unstained. The stainable droplets were easily extracted by acetone and pyridine.

After fixation for 48 hours, the lipid droplets of the glomerulosa and outer portion of the fasciculata stained heavily and uniformly.

FIG. 1. Section through the liver and bile duct of a rat. The tissue was fixed in Rossman's picric-acid-alcohol-formalin mixture. The deparaffinized section was oxidized in 1% periodic acid for 5 minutes and then stained with the Ashbel-Seligman reagents. Mucus stained intensely, the hepatic glycogen (bottom of figure) moderately.

FIG. 2. Section through the esophagus of a rat. The tissue was fixed in Zenker's fluid. The deparaffinized section was hydrolyzed in *1N* hydrochloric acid for 30 minutes at 60°C., and stained by the Ashbel-Seligman method. Nuclei were stained in a fashion comparable to that seen after the Feulgen reaction. In the upper left hand portion deeply stained elastic fibers may be seen. × 600.

FIG. 3. Section through the kidney of a rat. The tissue was fixed in 10% formalin for 1 month. The section was stained with the Ashbel-Seligman reagents without any preliminary hydrolysis or oxidation. The diffuse reaction of the convoluted tubules is absent in tissue fixed for short times. The material responsible for the reaction is not extractable in acetone. × 300.

FIG. 4. Elastic tissue in a small artery near the adrenal gland of a rat. The tissue was fixed for 24 hours in 10% formalin and the section stained with the Ashbel-Seligman reagents. × 600.

In the deeper portion of the fasciculata and in the reticularis, the droplets stained less regularly and less intensely. In addition to the droplets, the background cytoplasm took on a faint and diffuse tint throughout the adrenal cortex. Extraction in acetone or pyridine abolished the stain of the lipid droplets, but did not affect the diffuse cytoplasmic stain. The medulla was completely negative.

Longer periods of fixation (1 week to several months), caused a progressive enhancement of the background, lipid-insoluble, staining. Indeed, in sections fixed 2 weeks or longer, the background stain was so intense that the lipid droplets could be seen only with difficulty (Fig. 6). Nevertheless, when they could be distinguished, they were extractable with acetone or pyridine whereas the cytoplasmic background stain was unaffected.

In some other tissues, a similar diffuse, acetone- and pyridine-insoluble staining occurred after prolonged fixation in formalin. The isotropic segments of muscle stained quite intensely whereas the A discs were less deeply colored. The myelinated sheaths of peripheral nerves reacted intensely in a reticulated pattern, the appearance of which suggested the neurokeratin network as demonstrable by other histological methods. Elastic fibers were regularly and intensely colored (Fig. 2 and 4). Hepatic cells, especially those located in the central portions of lobules, reacted moderately. The convoluted segments of the renal tubules stained moderately or intensely (Fig. 3), whereas glomeruli stained only faintly and the medullary rays were uncolored.

The lipid soluble droplets of the adrenal cortex stained, but less intensely, with Schiff's leucofuchsin reagent. The background, diffuse lipid-insoluble stain was less intense and therefore less troublesome with most of the tissues enumerated above. Nevertheless, careful study revealed a similar background reaction in all of the locations, especially in tissues kept in formalin for long periods of time. Consequently, the Ashbel-Seligman reaction and the leucofuchsin procedure may be equated in that they stain similar structures under similar conditions. There are only minor differences in staining intensity.

Glycogen and mucopolysaccharides. Glycogen and mucopolysaccharides were investigated in the liver and tracheal-esophageal region. After oxidation in periodic acid, hepatic glycogen gave a strongly positive Schiff reaction but was stained only moderately by the Ashbel-Seligman technic (Fig. 1). Mucus, present in bile ducts and esophageal glands, reacted intensely by both methods. The matrix of

FIG. 5. Drawing of a section from the adrenal cortex of a rat after fixation in 10% formalin for 24 hours. The droplets in the adrenal cells are rapidly extractable in acetone. 40 × obj., 7 × ocular

FIG. 6. Drawing, similar to that shown in Fig. 5 from an adrenal fixed for 2 weeks in formalin. Note the increased background reaction which is insoluble in acetone. The reactive droplets remain acetone soluble. 40 × obj., 7 × ocular

cartilage in the tracheal rings was rather more deeply stained by the Schiff than by the Ashbel-Seligman procedure. The superficial layers of the esophageal epithelium, comprising the granular layer and the desquamating cells, contain substances which react with the periodic acid-Schiff reagents in a fashion characteristic of mucopolysaccharides. These regions stained equally well with either method.

Desoxypentosenucleoproteins. Sections of liver and esophagus were prepared after Zenker fixation. These sections were immersed in 1*N* HCl at 60°C. for 5, 15 and 30 minutes, after which they were exposed to leucofuchsin (Feulgen reaction) or to the Ashbel-Seligman reagents. The cell nuclei were stained similarly by both procedures, but the intensity of color was stronger after the Feulgen method.

Discussion

The rationale for the use of Schiff's leucofuchsin reagent in histology comes from the fact that it becomes recolored when reacted with aldehydes. However, the chemistry of this reaction is obscure, and detailed knowledge of the reaction products is lacking. The present observations, indicating that hydrazine reacts with the same structures which recolorize leucofuchsin, support the hypothesis that carbonyl groups form the site of reaction, because of the well-known formation of hydrazones from carbonyl compounds.

For demonstrating the lipid droplets of the adrenal cortex, the Ashbel-Seligman procedure is superior to the leucofuchsin method in that the droplets are more intensely stained and, if the proper duration of fixation is employed, the contrast between the droplets and the staining of the cytoplasmic background is greater. Similarly, for elastic fibers and peripheral nerve, the Ashbel-Seligman method is the preferable one. Mucus is stained satisfactorily by either method. On the other hand, glycogen and the matrix of hyaline cartilage are colored deeply by the leucofuchsin procedure and only faintly by the hydrazine method.

The various substances of tissues can be characterized to some degree by virtue of the procedures necessary to render them reactive to carbonyl reagents. Elastic fibers react immediately and directly with no previous treatment or hydrolysis. The plasmalogens are unreactive in their native form, but are stainable after short exposure to $HgCl_2$ (cf Hayes, 1949). The carbohydrates of glycogen and glycoproteins become reactive only after oxidation in periodic acid or other oxidants (Lillie, 1948). Desoxypentoseneucleoprotein is split by acid hydrolysis to form an aldehydic compound which is reactive (Feulgen and Rossenbeck, 1924). The present observations indicate that the lipid droplets of the adrenal cortex are unreactive in the fresh state and become reactive after fixation in formalin. Moreover, prolonged fixation in formalin causes the appearance of an acetone-insoluble reactive substance which is widespread in its occurrence.

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A NON-VACUUM FREEZING-DEHYDRATING TECHNIC FOR HISTOLOGY, AUTORADIOGRAPHY AND MICROBIAL CYTOLOGY

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ABSTRACT.—**A method for the freezing-dehydration of tissue and microbial cells is described which circumvents the need for high vacuum for the purpose of removing water from the cells by direct vaporization of ice. The present method utilizes the dissolution of ice from frozen tissue and cells by immersion in chemical dehydrating agents which remain liquid at low temperatures. Choice of the particular agent depends upon the desired result. Glycols are recommended if fixation is to be avoided, or water-soluble isotopes retained in the cells; alcohols or acetone are used if fixation is not undesirable. Specific applications of the method are given for use with tissues and with microbial cells. It has been demonstrated that radioactive phosphorus (P^{32}) is not leached out of the tissues during the procedure.**

Maximum retention of structure and substance of cells for cytologic or autoradiographic study is achieved with the freezing-drying technic of Altmann and Gersh (Bensley and Bensley, 1938; Holt, Cowing and Warren, 1949; Winteringham, Harrison and Hammond, 1950). This high vacuum procedure removes the intracellular water after instantaneous freezing by passing directly from the frozen to the vapor state. It requires expensive and complicated apparatus; is time-consuming (short-lived isotopes); requires embedding at 55°C. in paraffin, which must subsequently be removed; and is not applicable for routine study of a large number of specimens. Russell et al. (1949) have recently suggested the possibility of dissolving the ice from frozen tissue by immersion in cold alcohol. In the present method this principle is applied after instantaneous freezing of the tissue, and the water (as ice) is removed by continued immersion in dehydrating agents which remain liquid at the necessary low temperatures. Choice of the particular agent depends on the desired result. Glycols are recommended if fixation is to be avoided or water soluble radio-isotopes retained; alcohols or acetone may be used if fixation is not undesirable.

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A. Procedure for non-vacuum freezing-drying of tissues.

1. Fresh animal tissues cut in slices 2–3 mm. thick are placed directly into cold propylene glycol stored in a deep freeze below −20°C.
2. After a minimum of 1–2 hours at the low temperature, the tissue is transferred to a mixture of polyethylene glycols (Carbowax®) at 55°C. for infiltration.—This embedding mixture contains 9 parts Carbowax® 4000, and 1 part Carbowax® 1500. (Blank, 1949; Blank and McCarthy, 1950).
3. The tissues are infiltrated for 2–3 hours and blocks are prepared with this water-soluble wax.

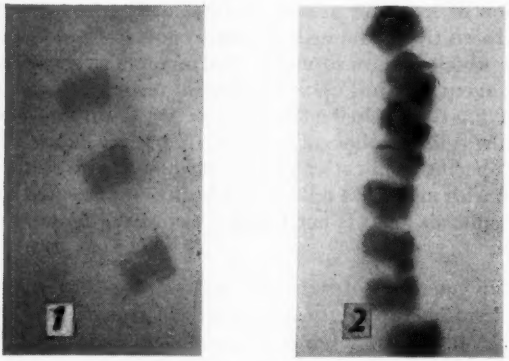

FIG. A. Autoradiograms of P^{32} in sections of same mouse liver, exposed for same period and developed simultaneously.
1. Formalin fixation and routine paraffin embedding method of preparing sections.
2. Unfixed, dehydrated in cold propylene glycol and embedded in polyethylene glycols. (See text)

4. Sections are cut in ribbons 2 μ or more thick, and floated onto slides as previously described (Blank, 1949; Blank and McCarthy, 1950).
5. The slides are stained with routine or special stains but, as with unfixed frozen sections, the time of staining must be reduced by about 80%.
6. The sections are then dehydrated to balsam, or mounted in water soluble media for lipids in the routine fashion.

B. Application of non-vacuum freezing-drying dehydration method for autoradiography to prevent leaching of water soluble substances. The method for this procedure is the same as outlined above through step 3, and is then modified as follows:

4. Individual sections are cut from the block after applying a thin layer of molten wax to the block face before each knife tra-

verse (Holt, Cowing, and Warren, 1949; Winteringham et al., 1950).

5. The wax reinforced sections are pressed directly against the sensitive emulsion to make the autoradiograph. After a suitable exposure, the emulsion is developed and fixed.

Table 1 summarizes the results of experiments to measure the leaching of radioactive phosphorus from tissues during various histologic procedures. Mouse liver made radioactive by intraperitoneal injection of phosphorus^{32} one day before, was divided into a number of samples. After each stage in the processing of the specimens (immersion in glycol, fixation, and embedding), aliquots of the fluids and the samples of the tissue were digested and prepared for counting on planchets in an end window Geiger-Müller counter. All radio-

TABLE 1. LEACHING OF RADIOACTIVE PHOSPHORUS DURING HISTOLOGIC PROCEDURES

	Cold (−20°C) propylene glycol for 12 hours	Formalin fixation for 12 hours	Bouin's fixation for 12 hours	Physiological saline for 2 hours
Loss of activity in initial fluid	0%	42%	43%	40%
Additional loss of activity by embedding in:				
Carbowax	0%	0%	—	—
Paraffin	—	37%	—	—
Total activity lost in embedding				
Glycol plus Carbowax	0%			
Fixation plus Alcohols plus Paraffin		79%		

activity in each piece of tissue was accounted for in the digested specimens plus the several fluids. It is apparent that routine methods of preparing histologic sections remove 4/5 of the radioactive phosphorus present, in contrast with the glycol method which removes practically none. (See Fig. 1)

C. Cytology of bacteria and fungi.

Application of this non-vacuum dehydrating principle has made it possible to prepare permanent cleared mounts of micro-organisms which reveal hitherto unobtainable cytologic detail (DeLamater and Hunter, 1951). Wet touch preparations of the organisms on cover slips are fixed in osmic acid vapor and stained by one of several methods (DeLamater et al., 1950; Smith, 1950).

After staining, rinsing in distilled water, and draining off the excess water, the procedure is as follows:

1. Immerse cover slip in absolute alcohol (or acetone) which is maintained at a temperature below −20°C. by packing in solid carbon dioxide.
2. After a minimum time of 12 hours in this cold solution, the cover slips are transferred to absolute alcohol at room temperature for not over 15 seconds.
3. They then are passed through 2 changes of xylene for 3 minutes each, and are mounted in any desired mounting resin.

Results of the method are shown in the accompanying figures.

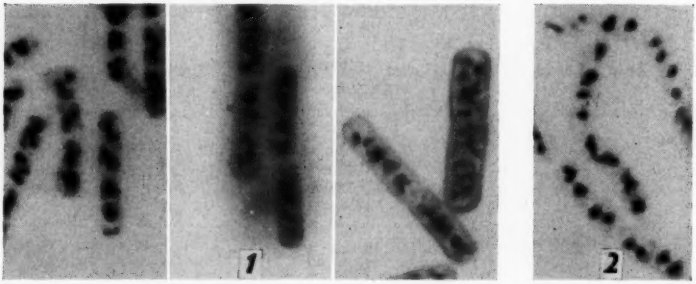

FIG. B. Stained smears of *Bacillus megatherium*

1. Bacterial cells dehydrated by non-vacuum freezing technic, showing nuclei in varous stages of activity, with interphase chromosomes as delicate beaded threads and telephase chromosomes as dense granules. × 3000.
2. Bacterial cells dehydrated at room temperature, showing marked shrinkage and distortion of intracellular components which obliterates chromosomal detail. × 3000.

CONCLUSIONS

A technic is presented which applies a new principle of freezing-drying cells for microscopic study with many advantages over current methods. It is simple, rapid, economical, and readily available. The examples (Fig. 2) indicate the amount of cytologic detail obtained and the retention of water-soluble cellular components, such as radioactive phosphorus. Detailed reports of further applications of this technic to histochemistry, autoradiography, and electron microscopy of tissues, and to the cytology of micro-organisms will appear subsequently.

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A STAINING AND DEHYDRATING PROCEDURE FOR THE HANDLING OF MICROÖRGANISMS[1]

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ABSTRACT.—**A new staining and dehydrating procedure for microorganisms is presented. It is based on the observation of Atwood and Orinstein (1949) that azure A or thionin, in the presence of SO_2, stains chromosomes intensely and apparently selectively. The staining process is as follows: Cover-slip smears are hydrolyzed in 1 *N* HCl at 60°C. for the previously ascertained optimal hydrolysis time. They are then briefly rinsed in distilled water and transferred to either of the two following stains, (1) 0.25% thionin (10 ml.) to which thionyl chloride (1 drop), as a source of SO_2, is added; (2) 0.25% azure A (10 ml.) to which thionyl chloride (2 drops), as a source of SO_2, is added. Neither of these stains decolorizes, i.e., forms a leuco base, in the presence of SO_2. Staining is allowed to proceed for a minimum of two hours.**

Following staining, the preparations are rinsed in distilled water, drained on filter paper, and immediately immersed in absolute alcohol which has been previously chilled to about −50°C. by packing in solid carbon dioxide. Preparations are left in this freezing alcohol for at least twelve hours, during which time dehydration proceeds. They are then passed through absolute alcohol at room temperature, through two transfers of xylene, and mounted in one of the mounting resins. The principle of this dehydrating procedure is presented by Blank et al. (p. 193 of this issue). Distortion is avoided by instantaneous freezing.

INTRODUCTION

Recent developments in the cytology of bacteria have depended largely upon the application of methods due to Piekarski (1937, 1949) who was one of the first to apply the Feulgen reaction to the study of bacteria with useful cytological results. To increase the distinctness of the normally very faint reaction, Piekarski (1937) suggested the use of Giemsa's stain in the place of the Schiff reagent following hydrolysis with *N* HCl at 60°C. He and his followers originally dehydrated and embedded their preparations in the conventional manner. Later many abandoned this method in favor of water mounts, Robinow (1945), Klieneberger-Nobel (1950), Smith (1950). Giemsa-stained water mounts show much detail but they suffer from several shortcomings. They are shortlived. The stain is not as specific as the

[1]This study was supported in part by U. S. Army Grant #W-49-007-MD-473.

Feulgen reaction and difficulties were experienced in making undistorted, dehydrated, permanent preparations of such material.

The present paper describes a new staining technic which is intense and selective, and a new procedure by which preparations so stained can be dehydrated and embedded in resins without distortion. In these procedures two essential changes in method are made: (1) new stains have been chosen which give more specific reaction for deoxyribosenucleic acid than the Giemsa stain, and which give a denser, more precise image to be observed than that produced by the Feulgen stain; (2) a method is introduced which permits the making of permanent, cleared, hardened mounts which retain the essential structure of untreated cells. This method applies a new principle of freezing-drying, as described by Blank, McCarthy and DeLamater (1951). Comparative photographs of bacterial cells dehydrated at room temperature and in the cold are presented in this paper.

Materials

The dyes used in these studies are: (1) thionin, Cat. No. 920, Certification No. NT16, of the National Aniline Division, Allied Chemical & Dye Corporation; (2) azure A blend, Cat. No. 136, Harleco.

Procedure

Fixation and Hydrolysis: Bacteria or yeasts are grown for any desired interval on agar plates. Blocks of agar containing surface growth of the organism are then cut out and placed upright on a small glass square. These are placed for purposes of preliminary fixation in osmic acid vapor in a closed chamber for five minutes. (The optimal period of fixation varies somewhat for each organism.) The agar block is then removed. Sections of it are cut off with a scalpel and inverted on cover slips. Impression or streak smears are then made. These cover-slip preparations are immediately placed (without drying) in a hydrolyzing bath of 1 *N* HCl at 60°C. for six minutes. (The period of optimal hydrolysis varies, depending upon the species of the organism, the phase of the organism in its growth cycle, and the media upon which it has been grown. Optimal conditions must be worked out for each organism.) The cover-slip preparations are then rinsed briefly in distilled water and are transferred to the stain. Up to this point the method is the same as those utilized by Piekarski (1937, 1949); Robinow (1942); and DeLamater and co-workers (1950).

Staining: It has been demonstrated by Atwood and Orinstein (1949) that dyes, such as methylene blue A and thionin, are capable of forming a linkage with sulfurous acid in the same manner as the pararosanilines used in the Schiff reagent, except that the formation of a leucobase does not occur. Dyes so treated with sulfurous acid

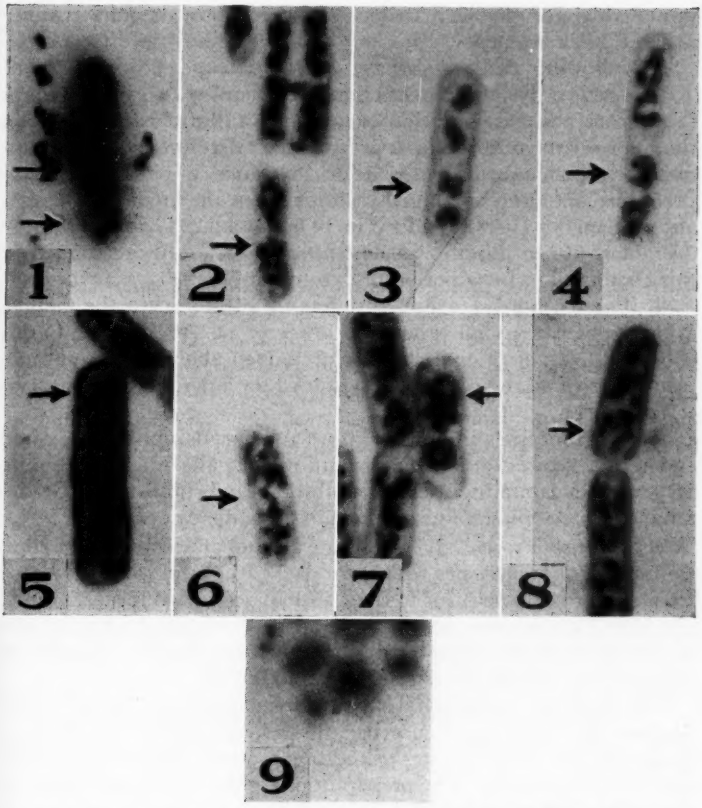

FIG. 1–8: *Bacillus megatherium;* Fig. 9: Micrococcus B–57; all photographs × 4850; FIG. 1–8 stained with thionin–SO_2; Fig. 9 stained with thionin alone.

FIG. 1 — A tetranucleate cell showing a typical metaphase spindle with polar bodies in the second nucleus from the top, and a polar view of the metaphase showing the three chromosomes in the upper nucleus.

FIG. 2 — Telephase separation of the daughter nuclei, showing the dense clusters of chromosomal granules and the persistent polar bodies toward which they are moving.

FIG. 3 — Early interphase polar bodies no longer visible.

FIG. 4 — Early interphase nuclei in which the nuclear membranes have reformed and within which the chromosomes are elongating into delicate threads.

FIG. 5 — Large interphase nucleus, showing nuclear membrane and the delicate chromosomal threads lying within. The nucleus is obviously surrounded by a layer of cytoplasm.

FIG. 6–8 — Large early prophase nuclei, showing the three contracting chromosomes becoming shorter and thicker.

FIG. 9 — Micrococcus B–57 — typical metaphase spindle in uninucleate cell showing polar bodies and clump of chromosomes.

presumably give specific reactions for aldehydes in the same manner as the Schiff reagent.

The following two stains have been used:

(1) The first dye, thionin, is a thiazin containing two amine groups in the para position. Addition of too much sulfurous acid to a solution of this dye will produce a precipitate. Therefore, careful adjustment of the amount of sulfurous acid required is necessary. In this procedure one drop of thionyl chloride, as in the method of Barger and DeLamater (1948), is added to 10 ml. of a 0.25% solution of the dye about fifteen minutes prior to use. Preparations are stained in this combination for a period of two to ten hours.

(2) The second dye, azure A, differs from thionin in the methylation of one para-amine group. To 10 ml. of a 0.25% solution of this dye, two drops of thionyl chloride are added about fifteen minutes prior to use, and the cells are also stained for a period of at least two to ten hours.

Whether one uses thionin or azure A depends upon the desired end result. Azure-A–SO_2 complex stains only the nuclei. Thionin-SO_2 complex contains an excess of thionin and stains the nuclei intensely, the cytoplasm and cell membrane a delicate violet.

Dehydration Procedures – Absolute alcohol or acetone is placed in Columbia staining dishes which are packed in solid carbon dioxide for a matter of hours prior to their anticipated use to insure optimal cooling. (Temperature must be below −20°C.) In our laboratory this is accomplished in a special airtight icebox in which 100 pounds of solid carbon dioxide is constantly maintained.

After staining, the preparations are rinsed briefly in distilled water, drained on filter paper, and placed immediately in the cold absolute alcohol or acetone. Freezing of the preparations under these conditions is instantaneous. The preparations are then left in this freezing menstruum for a period of at least twelve hours. They are then removed, placed for not more than fifteen seconds in absolute alcohol at room temperature, and transferred through two passages of xylene. They are then mounted in one of the various mounting resins.

Results – By application of these procedures it has been possible to obtain preparations in which the intrinsic structure of the nucleus of bacterial and other types of cells has not been disturbed and in which the specificity and intensity of staining of the chromosomal elements are such that optimal observations may be made. Figures 1–8 show the nuclear cycle of *Bacillus megatherium* in which a typical mitotic process has recently been observed (DeLamater, 1951), which will be reported in detail in the near future (DeLamater and Hunter, 1951; and DeLamater and Mudd, 1951). Figure 1 shows a typical metaphase spindle in which can be seen two polar bodies. A polar view of the metaphase stage, showing three haploid chromosomes is also to be observed in the same cell. Figure 2 shows a tele-

phase stage in the separation of daughter nuclei in which the centriole can still be seen. Figure 3 shows a very early interphase, while Figures 4 and 5 show the progressive enlargement of the interphase nuclei with the concomitant elongation of the chromosomes to delicate beaded threads. Figures 6–8 show the contraction of the chromosomes during prophase. Figure 9 shows a typical metaphase spindle in a Micrococcus, stained only with thionin following hydrolysis, and dehydrated in the manner described.

Summary

Two new modifications of cytologic methods useful in the study of micro-organisms are presented. The first of these consists of the selection of new stains which are apparently specific for deoxyribosenucleic acid. The second consists of a new dehydration technic which permits the making of permanent, cleared, undistorted preparations. Both developments permit the definition of nuclear phenomena in bacteria and related organisms which have heretofore escaped observation.

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THE PROBLEM OF A STANDARDIZED TECHNIC FOR THE METHYL-GREEN-PYRONIN STAIN[1]

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ABSTRACT.— **During the study of possible staining methods for the demonstration of pentose nucleic acid in tissue sections, two technics employing methyl green and pyronin were found to be satisfactory. Both of these are modifications of that originally proposed by Scudder. Methyl green, pyronin Y (no longer on the market), and pyronin B, all National Aniline certified dyes, were used. The results were compared with other technics. Numerous fixatives were tried, and Carnoy's fluid and absolute alcohol proved to be the most satisfactory. Frozen-dried tissue and tissue dehydrated in the frozen state from *N*-butyl alcohol also stained well. The problem of purification of methyl green is discussed. The effect of arbitrary change of pH of the staining solutions is described.**

In the recommended technic with pyronin B, 0.5 g. methyl green (purified by extraction with chloroform) is dissolved in 100 ml. of *M*/10 acetate buffer at pH 4.4. This solution is extracted repeatedly with further chloroform to remove residual methyl violet, and then 0.2 g. pyronin B is dissolved in it. Sections on slides are hydrated and stained in this solution for 3–5 min. They are rinsed and blotted; before they are completely dry, they are immersed in a mixture of 3 vol. tertiary butyl alcohol with 1 vol. absolute ethyl alcohol for 2 min. or longer. Sections are cleared in xylene and mounted in Clarite or Permount. Chromatin is stained blue-green by this technic, and nucleoli and cytoplasmic granules of liver cells are stained dark rose-lavender.

The study of nucleic acid metabolism in cells has been hindered because, although the Feulgen reaction is an apparently adequate method for the demonstration of desoxypentose nucleic acid (Stowell, 1945), the histochemical localization of pentose nucleic acid is not so easily accomplished. Both forms of nucleic acid react with basic dyes, and this fact has been utilized to reveal the site of pentose nucleic acid by Serra (1946) and others. In 1940 Brachet, using the pyronin-

[1]This investigation was aided by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

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methyl-green stain of Unna (1902), expressed the opinion that pyronin appeared to be a specific stain for pentose nucleic acid. He utilized ribonuclease, an enzyme said specifically to degrade pentose nucleic acid, to control his experiments. Subsequent investigators, notably Pollister and Ris (1947) have accepted this thesis and have published data which in their opinion tend to confirm it. In a recent article, however, Pollister and Leuchtenberger (1949) have said that pyronin is somewhat less specific than originally stated, but that methyl green appears to be specific for the staining of chromatin. Kurnick (1950 a,b) likewise agreed that methyl green appears to stain desoxypentose nucleic acid specifically.

The methyl-green-pyronin stain has been used also for the demonstration of plasma cells in tissue sections and as a counterstain for the Gram technic on smears of exudates. Although numerous modifications have been suggested, Scudder (1931a) and Hunt (1944) were the only investigators who recommended a return to the aqueous solution of the type originally described by Pappenheim (1899, 1901). An aqueous solution of the dyes was said to be more stable than one incorporating alcohol, glycerol and phenol (Unna, 1902) and was recommended as a counterstain for smears in the Gram technic and as a stain for formalin-fixed tissues (Scudder, 1931b; Lisa, 1938). In 1944 Scudder recommended dissolving 0.65 g. of methyl green and 0.1 g. of pyronin Y in 100 ml. of hot distilled water with storage for several days or weeks before using.

Materials and Methods

Solutions of dyes (Table 1) were prepared according to a number of methods and tested on tissues preserved in a variety of fixatives. Since Hunt (1944) and Pollister and Leuchtenberger (1949) said that methyl green purified by extraction with chloroform is superior to methyl green as sold by the manufacturer, methyl green was purified as follows: One to ten grams of the dye were shaken with a volume of chloroform ten to twenty times as great. The resulting suspension was filtered with suction and the process repeated twice or until the chloroform was colorless rather than lavender on filtration. The undissolved dye was dried and stored.

Solutions according to the Unna formula were made with commercial methyl green and pyronin Y, with purified methyl green and pyronin Y, and with purified Methylgrün and Pyronin. Solutions were made with methyl green and pyronin according to the formulas of: Conn and Darrow (pyronin B), Cowdry (pyronin Y), and Scudder (pyronin Y). One per cent solutions of purified methyl green, malachite green and brilliant green and a 0.5% solution of pyronin Y were made for use in the Hunt technic. Solutions according to the Scudder formula were made with: purified methyl green and pyronin Y, malachite green and pyronin Y, brilliant green and pyronin Y,

and with purified methyl green and pyronin B, acridine red, acridine orange, basic fuchsin, safranin O or rhodamine B replacing pyronin Y. A solution of malachite green and acridine red as suggested by Hitchcock and Ehrich (1930) was made. Finally a solution of methylene green and pyronin Y was made according to a formula of D. A. Johansen (personal communication).

Results were compared after differentiation and dehydration in the following five solutions: absolute ethyl alcohol, absolute alcohol and xylene in equal portions by volume, absolute acetone, isopropyl alcohol, and a mixture of three parts of tertiary butyl alcohol to one part of absolute ethyl alcohol (Stowell, 1942). It was found necessary to blot sections with smooth filter paper after rinsing and before dehydration so that all the dye would not be removed from the sections. Although differentiation could be accomplished with any of these reagents, the tertiary butyl alcohol mixture was most satisfactory because in this solution differentiation requires minutes rather than seconds.

TABLE 1. DYES USED AND THEIR SPECIFICATIONS

Dye	Company	Color Index Number	Certification Number, or Lot Number	Dye Content Per cent
Methylgrün	Grübler-Hollborn	?	H 172	?
Pyronin	Grübler-Hollborn	?	H 232	?
Acridine orange	National Aniline	788	11538	?
Acridine red	National Aniline	740	12277	?
Basic fuchsin	National Aniline	677	NF–56	97
Brilliant green	National Aniline	662	NBg–14	93
Malachite green	National Aniline	657	NMg–14	98
Methyl green	National Aniline	685	NG–26	85
Methylene green	Coleman & Bell	924	?	?
Pyronin B	National Aniline	741	NP–11*	?
			NP–13	32
			NP–14*	?
Pyronin Y	National Aniline	739	NP–10	48
Rhodamine B	National Aniline	749	11964	?
Safranin O	National Aniline	841	NS–21	95

*Furnished through the courtesy of Dr. Conn. Miss Scudder and Dr. Conn generously supplied samples of pyronin Y for comparative purposes.

Tissues, usually thin blocks of mouse liver (chosen because of the large quantity of basophilic cytoplasmic granules), were fixed in the following solutions: absolute ethyl alcohol; 10% aqueous, neutral formalin; chilled absolute acetone (Gomori, 1939); the alcohol, formalin and acetic acid mixture of Serra; Bouin's fluid with acetic acid; Stieve's fluid (Romeis, 1948, paragraph 333); Zenker's fluid; Helly's fluid; Regaud's fluid; and both types of Carnoy's fluids (six parts of absolute alcohol to three parts of chloroform to one part of glacial acetic acid; and three parts of absolute alcohol to one part of glacial acetic acid). Fragments of mouse liver were prepared by the

freezing-drying method of Stowell (1951). Small pieces of mouse liver frozen in isopentane chilled in liquid nitrogen were dehydrated at —40°C. in *N*-butyl alcohol. Most of the tissues, after washing when necessary, were dehydrated and cleared by a graded alcohol-cedarwood oil sequence. The acetone-fixed tissues were dehydrated by several changes of acetone and were treated with chloroform prior to paraffin infiltration. The frozen-dried tissues were infiltrated directly with paraffin under vacuum. The butyl alcohol dehydrated tissues were allowed to come to room temperature in the third change of *N*-butyl alcohol after six days at —40°C. Then they were immersed in a solution of equal parts of melted paraffin and *N*-butyl alcohol at 54° C. for 15 minutes. Infiltration with three changes of paraffin under vacuum at 54°C. was done for 30 minutes. All tissues were embedded in paraffin-beeswax mixtures and sectioned at 6 μ.

On repeated examinations during months of use it was found that solutions of methyl green and pyronin Y (Scudder, 1944) had a pH of from 4.3 to 4.4. The stain was also made up in acetate and phosphate buffers varying from pH 3.0 to 8.0 in steps of 0.5 pH units. Since the best results with both buffered series were obtained in sections stained at pH 4.0 and 4.5, it did not seem advisable to buffer or alter the natural pH of the staining solution.

At this stage of the investigation it was found that pyronin Y was not commercially available. No other dyes, even those structurally similar to pyronin Y (Conn, 1946), had proved satisfactory in combination with methyl green in simple aqueous solution. The pH of staining solutions of pyronin B (NP–11, NP–13, and NP–14), acridine red and rhodamine B in combination with methyl green ranged from 3.4 to 3.9. The pH of these solutions was raised to 4.4 by the addition of M/10 acetate buffer. Sections stained with each lot of pyronin B and methyl green at this pH were most satisfactory.

An experiment similar to that described above was made with solutions in M/10 acetate buffer of purified methyl green and pyronin B (NP–13), acridine red and rhodamine B. Sections of mouse liver fixed in absolute alcohol and Carnoy's fluid (with chloroform) were stained at pH 3.0, pH 3.5, pH 4.0, pH 4.5, pH 5.0 and pH 6.0. In the solution with pyronin B, satisfactory differential staining was obtained at pH 4.0 and pH 4.5, but with acridine red and rhodamine B no improvement over the results obtained with unbuffered aqueous solutions could be detected.

Kurnick (1950,b) recommended the extraction of an aqueous buffered solution of methyl green with chloroform to effect removal of contaminating methyl violet. The fact that methyl violet is present in solutions of methyl green purified in the dry state with chloroform was noted when it was found that thyroid colloid stained lavender from a solution of purified methyl green and water and from a solution of purified methyl green in the mixture of Unna. It was found

that all the methyl violet can be removed from aqueous solutions of methyl green by repeated extractions with chloroform. However, if such a solution is allowed to remain at room temperature for two weeks, methyl violet again becomes detectable on extraction with additional chloroform.

Results

If pyronin Y is available, the following modification of Scudder's technic gives most satisfactory results: 0.5 g. purified methyl green and 0.1 g. pyronin Y are dissolved in 100 ml. hot distilled water. The solution is stored in amber glass-stoppered bottles. It may be used as soon as it cools.

Sections on slides are hydrated and immersed in the staining solution for three to five minutes. The sections are rinsed in distilled water for a few seconds and then are blotted with several thicknesses of smooth filter paper. Before the sections have completely dried, they are immersed in the tertiary butyl alcohol mixture for at least two minutes with occasional agitation. The slides are passed through two five-minute changes of xylene and mounted in one of the artificial resins such as Permount or Clarite.

With pyronin B the above technic was used, but the staining solution was modified as follows: 0.5 g. methyl green (purified in the dry state by extraction with chloroform) is dissolved in 100 ml. of M/10 acetate buffer at pH 4.4. This solution is extracted repeatedly in a separatory funnel with chloroform until the latter is not colored. Usually three extractions with 30–40 ml. of chloroform are sufficient. The pyronin B (0.2 g) is dissolved in the methyl green solution. It may be used immediately.

The best stains of sections of mouse liver were obtained with fixation in absolute alcohol and in Carnoy's fluid (with chloroform). Tissues prepared by freezing-drying or by dehydration in *N*-butyl alcohol also stained well. Liver fixed in Carnoy's fluid (without chloroform) stained equally well, but was too brittle. Tissues fixed in chilled acetone stained well, but cytologic detail is poor, and it likewise is not recommended. Liver fixed in 10% formalin and in Serra's fluid stained moderately well but more diffusely with pyronin than after fixation in Carnoy's fluids. Liver fixed in Bouin's, Zenker's and Regaud's fluids was not stained satisfactorily. Liver fixed in Helly's fluid was diffusely stained by pyronin as was that fixed in Stieve's fluid, and the chromatin failed to retain much of the methyl green.

The technics and staining solutions different from the ones finally selected were always tested with sections of liver fixed in absolute alcohol and 10% formalin and usually in a number of other fixatives. Sections stained with the Unna type formula and modifications, no matter whether with purified, National Aniline or Grübler-Hollborn dyes, were generally diffusely pink or red. Although consider-

able detail was apparent, they were inferior to sections stained with aqueous solutions. Sections stained with the Hunt technic using purified methyl green and pyronin Y were satisfactory, but less brilliant than with the Scudder technic. Sections stained with malachite green, brilliant green and methylene green were not satisfactory. The green was not confined to nuclear chromatin. Sections stained with the Hitchcock and Ehrich technic had well stained nuclei, but the cytoplasmic stain was indefinite.

In sections of mouse liver fived in Carnoy's fluid (with chloroform) and stained with the modified Scudder formula of purified methyl green and pyronin Y, the nuclear chromatin is finely divided and blue-green in color. The cytoplasmic granules and nucleoli of liver parenchymal cells are clear rose-pink. Basement membrane, bile duct epithelial cytoplasm and endothelial cytoplasm are very pale pink-tan or colorless and barely discernible. Red blood cells are refractile and yellow. Plasma cells have brilliant rose-red cytoplasm.

When the buffered solution of pyronin B and methyl green is used on similarly fixed tissues, nuclear chromatin is also colored blue-green. Nucleoli and cytoplasmic granules are dark rose-lavender. Little coloration of other tissue components in mouse liver can be detected. The cytoplasm of plasma cells stains red-purple.

The results of staining a variety of tissues with the methyl green and pyronin Y solution are reported in detail in another paper (Taft, 1951). With the buffered solution of methyl green and pyronin B, color differentiation is generally as satisfactory, although slight differences in hues are evident.

Discussion

One of the basic problems in this technic has been that these dyes, particularly methyl green, are easily removed from tissue sections during dehydration and differentiation. The suggested use of alcohols of higher molecular weights provides satisfactory, slower differentiation. The simple technics described in this paper gave consistently better and more readily reproducible results than other technics tried. Among other advantages, the most important is that the background stain, which has concerned other authors (Pollister, 1949) who have used these technics, seems to be avoided almost entirely with pyronin Y and, to a somewhat lesser extent, with pyronin B. In contrast to toluidine blue and azure A, both of which have been recommended for the demonstration of cytoplasmic basophilia, the use of an additional technic to demonstrate chromatin is avoided, and although metachromasia is prominent, pyronins do not give as much background color as either of these other two basic dyes.

The observation that methyl violet appears in an aqueous solution of methyl green despite purification in solution by extraction with chloroform, and even from the solution of Unna which is said

to prevent degradation of methyl green, reveals that this dye is not as stable in solution as desired and as was thought by Scudder. When utmost precaution is necessary, the staining solution should be made fresh and used for only a few hours after preparation.

When this study was undertaken, it was hoped that a staining technic could be devised which would be simple and applicable to sections of tissues fixed in a variety of ways. A part of this aim has been achieved, in that a relatively simple technic for performing this stain has been developed which gives reproducible results. Its main drawback for routine use is that best results in mammalian tissues are obtained only by less routinely employed methods of fixation. Tissues fixed in absolute alcohol, although generally satisfactory, have a marked fixation gradient which results in artifacts on the periphery of sections and at times areas of irregular staining within the sections. There is more background stain in tissues fixed in formalin, though never as much as in sections stained with the Unna type of solution. For this reason, although formalin fixation is perhaps adequate for survey purposes, it is unsatisfactory for the accurate localization of substances which may be demonstrated by these dyes.

The use of this staining solution in the localization of pentose nucleic acid in tissue sections is discussed in another paper (Taft, 1951). However, if any combination of methyl green and pyronin is to give satisfactory results in this type of histochemical localization, the technic with methyl green and pyronin B described in this paper may be the most applicable.

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A RAPID METHOD FOR PREPARING PARAFFIN SERIAL SECTIONS OF TEETH AND THEIR SUPPORTING STRUCTURES IN THE DOG[1]

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ABSTRACT.—**A method of preparing dogs' teeth for serial sectioning involving the following steps is described. The tissues are fixed in 10% formalin followed by partial dehydration in 80% iso-propyl alcohol; decalcified in 5% nitric acid in 10% formalin; washed for 5–8 hours in running water and neutralized in 5% aqueous sodium sulfate for 24 hours, followed by further washing in running water for another 24 hours. They are then dehydrated over a period of 4 days in increasing grades of iso-propyl alcohol and embedded in tissue-mat in sub-atmospheric pressure of 10 lb. Sections so obtained were better suited for cytological study than celloidin preparations, and serial sections could be obtained in a much shorter time.**

Routine paraffin impregnation and embedding has been successfully applied in this laboratory, to teeth, bone and soft tissue. Various technics, notably those involving the use of celloidin as an impregnating medium have been employed for the sectioning of teeth. During the course of studies in periodontal research in this laboratory (Linghorne and O'Connell, 1950), it was necessary to prepare thin serial sections of large numbers (over 100) of teeth, bone and soft tissue suitable for cytological study. As the celloidin technic is not only slow, particularly for serial sectioning, and also provides material less suited for cytological observations than is the case with thin paraffin sections, it was found essential to devise a paraffin technic which would fulfil these needs, i.e., to obtain consistently satisfactory sections as thin as 5μ at intervals of 250μ. Such technics have already been described by Gairns (1944) who devised a paraffin method for developing and mature teeth in man, and also by Morse (1945). The latter technic, using *N*-butyl alcohol in the dehydration schedule, was almost as lengthy as that using celloidin. After several preliminary trials with ethanol, xylene and chloroform which hardened the blocks excessively, a slower, and consequently more complete dehydration technic was evolved, beginning with 50% iso-propyl alcohol and increasing, in stages, to 100% iso-propyl alcohol. Blocks so treated cut fairly easily in any plane, and sections of 10μ thickness or

[1]This work was supported by a grant from the National Research Council of Canada.

less were obtained from most. In a least 50% of the cases, sections formed unbroken ribbons for serial cutting.

Staining with hematoxylin and eosin, although inadequate in some cases, was satisfactory in most. The factors responsible for poor staining have not been determined, but are under investigation at the present time.

Method

The jaws of the dogs were completely fixed in 10% formol-saline (approx. 5 days) after which they were transferred to a decalcifying solution of 5% nitric acid in 10% formalin. When soft enough to permit trimming, the canine teeth, on which the experiments were being conducted, together with as much surrounding tissue as possible, were removed. These in turn were placed in fresh nitric acid formalin solution until they were completely decalcified, when they were trimmed to the required size. During the treatment of the specimens at all stages, care had to be taken in handling etc. in order to ensure that separation of the gingiva from the tooth did not occur, thereby giving rise to artifact in the subsequent sections. The specimens were washed in running water for 6–8 hours after which they were neutralized in a 5% aqueous solution of sodium sulfate for 24 hours. This was followed by washing in running water for another 24 hours.

Dehydration schedule was as follows:

50% isopropyl alcohol	9 a.m.	1 p.m.	1st day
70% isopropyl alcohol	1 p.m.	5 p.m.	
80% isopropyl alcohol	5 p.m.	9 a.m.	2nd day
90% isopropyl alcohol	9 a.m.	5 p.m.	
95% isopropyl alcohol	5 p.m.	9 a.m.	3rd day
100% isopropyl alcohol	9 a.m.	5 p.m.	
100% isopropyl alcohol	5 p.m.	9 a.m.	4th day
100% isopropyl alcohol .(50°C.)[2]	9 a.m.	10 a.m.	

Blocks were transferred to molten tissue-matt (M.P. 54°–56°C.)[3] in the vacuum embedding oven at a temperature of 58°–60°C. for a period of 4 hours, followed by a similar length of time in fresh tissue-matt. A sub-atmospheric pressure of 10 lb. was maintained in the oven during impregnation.

Sections, when cut, were floated from warm (50°C.) 0.25% aqueous gelatin on to clean microslides. Each section was fixed to the slide by placing it in a closed dish, at the bottom of which was a layer of formalin. The dish was placed in the paraffin oven at 50°C., where

[2]Iso-propyl alcohol acts as a clearing agent when heated, thereby eliminating the use of xylene, chloroform or any other of the commoner agents which tend to harden tissue to a greater degree.

[3]Fisher Scientific Co., Montreal, P.Q.

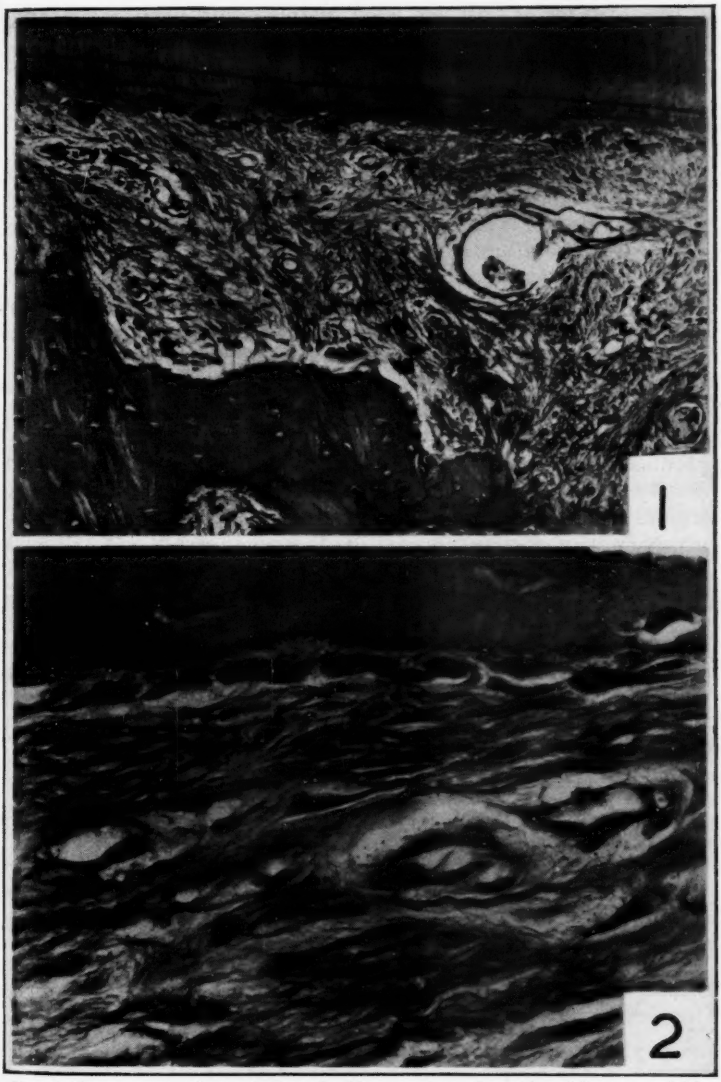

FIG. 1. Low power photomicrograph. Note preservation of architecture which shows the arrangement of dentine, cementum, periodontal membrane and living and dead bone. Hematoxylin & Eosin × 200

FIG. 2. High power photograph showing new cementum and new periodontal membrane. Cytological detail of cementocytes and newly differentiated cementoblasts is well delineated. × 1000
Hematoxylin and Eosin.

The tissue from which this material was prepared was made available by the courtesy of Dr. W. J. Linghorne, Banting and Best Department of Medical Research, University of Toronto.

it remained for at least one hour. Staining with hematoxylin and eosin was carried out in the usual manner. It was found that if reasonable care was exercised when transferring slides from one solution to another, and if the stained preparations were mounted as soon as possible from xylene, the sections adhered firmly to the glass slides.

The above are the times employed in this department but these may be altered to suit the individual requirements of other laboratories, care being taken, however, that the length of time in absolute iso-propyl alcohol is not appreciably lengthened.

Discussion

For cytological study, paraffin sections of teeth (Fig. 1 & 2), obtained by the method above, were superior to any which had been produced in this laboratory by celloidin technics, for in common with sections of any tissue cut after paraffin impregnation, thinner and clearer preparations could be stained than was possible when celloiden was employed. This method offers possibilities of rapidly producing, in some quantity, sections of even large canine teeth. Serial sectioning, after celloidin impregnation, is slower and more tedious compared with the method just described, especially if it is necessary at times to cut through 10–12 mm. of each block. Staining is facilitated, for the slides may be processed in batches held in staining racks.

Acknowledgment

The author wishes to express his appreciation of the advice and helpful criticism offered by Dr. W. Stanley Hartroft throughout this work.

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CASON'S POLYCHROME STAIN FOR CELLOIDIN EMBEDDED MATERIAL

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Received for publication Jan. 10, 1951

Cason[1] recently reported a rapid one-step modification of the Mallory-Heidenhaim connective tissue stain which has proven excellent for routine work because of its simplicity and uniformity. However, the Cason modification was described for paraffin-embedded material. With celloidin blocks, used widely in neuropathology, there are two problems to be overcome in using a single polychrome stain: first, a selection of a differentiating fluid which acts uniformly; second, a method of dehydration and clearing which will not affect the clarity of the stain or produce cracking in the tissue.

An anilin-95%-alcohol solution is employed to give an evenly differentiated polychrome stain. Staining time can be decreased from that stated in Cason's modification because of the greater thickness and absorptive properties of celloidin. The sections are agitated in the differentiating fluid until the desired amount of decolorization has been obtained and the celloidin background is clear of all stain. Chloroform is added to the differentiating fluid to prevent any solvent action on the celloidin. A subsequent wash in the 95% alcohol with chloroform removes traces of anilin oil and stops decolorization. Sections may be examined microscopically from this fluid and returned to the anilin alcohol solution if differentiation is not complete. The 95% alcohol also acts as a dehydrating agent for the tissue and thus differentiation and dehydration are accomplished simultaneously. The sections are next transferred directly to a clearing solution of terpineoil and xylene, averting the use of absolute alcohol which tends to dissolve the celloidin. Sections are mounted from a pure xylene solution which completes clearing.

The staining solution was exactly the same as employed by Cason as follows:

STAINING SOLUTION

To 200 ml. distilled water, add the contents listed below one by one, stirring to dissolve the mixture with each addition:

Phosphotungstic acid crystals (Merck)	1 g.
Orange G (C. I. No. 27)	2 g.
Anilin blue, W. S. (C. I. No. 707)	1 g.
Acid fuchsin (C. I. No. 692)	3 g.

[1]CASON, J. E. 1950. A rapid one-step Mallory-Heidenhaim stain for connective tissue. Stain Techn., **25**, 225–6.

Procedure

Celloidin embedded material cut at 15 μ.

1) Wash sections in distilled water.
2) Stain for 15 to 20 *seconds,* agitate sections.
3) Wash quickly in distilled water.
4) Transfer directly to differentiating fluid:

95% alcohol	85 ml.
Fresh anilin oil	3 ml.
Chloroform	12 ml.

Agitate sections until celloidin background is clear of coloring. Change differentiating fluid with each new group of sections.

5) Wash in following solution to complete differentiation and to dehydrate sections:

95% alcohol	85 ml.
Chloroform	12 ml.

6) Clear in following solution:

Terpineoil	25 ml.
Xylene	75 ml.

7) Wash in xylene and mount in Fisher Permount.

Results

Large sections of neurological material were stained and decolorized without cracking and showed no change of homogeneous coloring appearance after prolonged exposure to bright light. The color affinity for tissue constituents was the same as obtained with the original Mallory technic.[2]

[2]Mallory, F. B. 1938. Pathological Technique. W. B. Saunders Co., Philadelphia.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

HOLT, MARGARET W., and WARREN, SHIELDS. **A radioautographic method for detailed localization of radioactive isotopes in tissue without isotope loss.** *Proc. Soc. Exp. Biol. and Med.*, **73,** *545–9.* 1950.

Animal tissues were frozen at –170° C. in isopentane, dehydrated and infiltrated with paraffin in vacuo, cut and mounted directly on a slide or cover glass. Paraffin was removed either by placing the mounts in an oven and allowing the melted paraffin to drain off or by means of a paraffin solvent. The solvent, when used, was allowed to evaporate in an oven before attaching the mounted section to a photographic plate. This method of handling prevented the loss of I^{131} ordinarily encountered when tissue was processed through aqueous media. Autographs were secured by mounting the sections facing the emulsion of the photographic plate but separated from it by a spacer ring surrounding the section. This ring of polystyrene plastic was 0.001 in. thick and provided an air gap of about 18μ between the surface of the section and the emulsion. The staining of the section and processing of the autograph were done separately after exposure and separation of the two components. Registration of fields was accomplished by making tiny crosses of radioactive ink on the slide or cover glass. The ink was prepared by adding carrier free P^{32} to Clay Adams Gold Seal black glass marking ink. The X marks were made by drawing an extremely fine glass capillary through dots of ink placed on the slide and before the ink dried. A microscope eyepiece fitted with a ruled micrometer disc was employed for checking positions which corresponded on the autograph and the stained section.—*H. A. Davenport.*

VETTER, J. P. **An inexpensive slide-area marker.** *J. Biol. Phot. Assoc.*, **18,** *180–1.* 1950.

The author suggests the use of a metal inking sleeve designed to fit snugly over a low power (10 ×) objective for marking in a circle of ink areas of histological slides to be photographed. When an area is to be marked, the operator lowers the objective covered by the sleeve to an inked plain glass slide. This improvised inking "pad" inks the margin of the lens-opening in the sleeve. After inking, the operator places the slide to be examined under the objective and when an area is located the sleeve is pressed against the field. In this manner, rings of ink are impressed upon the slide circling the areas to be photographed.—*Germain Crossmon.*

MICROTECHNIC IN GENERAL

DEMENT, JACK. **Immersion liquids and mounting media for fluorescence microscopy.** *Trans. Amer. Micr. Soc.*, **69,** *357–8.* 1950.

Most of the commonly used mounting media are fluorescent. This fluorescence can be quenched without appreciably changing the refractive index so that the following substances can be used: Cedar oil, by adding 0.05 – 0.7% nitrobenzene to small batches; paraffin oils and castor oil, by adding 0.05 – 0.1% nitrobenzene or 0.5 – 0.8% chloroform or carbon tetrachloride; plastics (Neville Clarite, Ward's bioplas or selectron), by adding similar amounts of nitrobenzene. Picric acid and trinitrotoluene also have quenching properties.—*Virgene Kavanagh.*

DYES AND THEIR BIOLOGICAL USES

MILLER, D. D., and ELLIKER, P. R. **A rapid method of testing for quaternary ammonium compounds in milk.** *J. Dairy Sci.*, **34**, *273–8.* 1951.

A method is described for the determination of quaternary ammonium compounds (QAC) in milk. The reagents are prepared as follows:

Indicator solution: (a) Dissolve eosin (dye concentration about 90%, C. I. No. 768) in acetone (analytical reagent) at the rate of 0.5 mg. of dye to 1 ml. of acetone. (b) Add acetone-eosin solution to tetrachloroethane (technical grade) at the rate of 1 ml. of acetone-eosin to 9 ml. of tetrachloroethane. (c) Remove the reddish color from the solution by adding citric acid crystals (monohydrate, analytical reagent) at the rate of 10 mg. of crystals to each ml. of dye solution. (d) Shake for 1 min. or until the red color disappears. (e) Filter through filter paper.

Buffer: Prepare a solution of citric acid (monohydrate, analytical reagent) at the rate of 25 g. to 100 ml. distilled water and adjust to pH 3.5 with 50% NaOH (analytical reagent). Approximately 12 ml. of NaOH to 100 ml. of citric acid solution usually are required.

Anionic solution: Prepare a 0.01% solution of active anionic compound from 10% Fisher Laboratory Aerosol (10% dioctyl sodium sulfosuccinate). This represents approximately a 1:1000 dilution of the 10% Aerosol. Other anionic surface active agents may prove suitable for this solution.

To proceed: (a) Place 1 ml. of the milk to be analyzed in a test tube. Add 5 ml. of distilled water, 1 ml. of indicator solution and 0.2 ml. of buffer solution. Shake vigorously for 10 sec.

(b) Centrifuge to separate solvent fraction from milk solids and water. This may be accomplished by centrifuging for 5 min. at 3200 RPM in a 10-in. centrifuge. A Babcock centrifuge can be used but may require as long as 25 min. for the same operation. Three distinct layers should be apparent following the centrifuging. The top layer should be a liquid; the middle layer should consist chiefly of precipitated protein; and the lower layer should consist of the solvent containing most of the QAC in the sample. The procedure up to this point may be employed as a qualitative test for detection of QAC in milk. Presence of a QAC is indicated by development of a red color in the solvent layer. A pink to red color usually indicates at least 5 ppm. QAC. Subsequent steps are necessary for an approximate determination of QAC present.—*W. G. Walter.*

ANIMAL MICROTECHNIC

GORDON, MORRIS A. **Rapid permanent staining and mounting of skin scrapings and hair.** *Arch. Dermat. and Syph.*, **63**, *343–6.* 1951.

The following *materials are required:* (1) solution of methylene blue hydochloride, prepared by dilution of 1 part of a saturated 95% alcoholic solution (1.48 g. per 100 ml.) of the dye with 9 parts of distilled water; (2) distilled water; (3) 1% solution of acetic acid; (4) 1,4-dioxane *(caution:* avoid excessive inhalation of dioxane vapors); and (5) a synthetic resin such as HSR (Hartman-Leddon Co., Philadelphia).

General procedure: (1) Scrapings (the thinner the better) or hairs are placed directly on a clean miscroscope slide and covered with 1 or 2 drops of methylene blue solution; (2) the dye is allowed to act for 30 seconds to 1 minute (longer for very thick specimens) and then is drained off by means of small torn pieces of bibulous paper. (3) Distilled water is applied with a dropper, permitted to remain until dye ceases to flow from the specimen, and drawn off, as above, to dryness. A second application of water may be necessary for removal of excess dye. (4) In some instances more vigorous decoloration is required for best results. For this

1% acetic acid is used and drawn off after an appropriate interval and this procedure followed by an application of water. (5) 1 or 2 drops of 1,4-dioxane are added to dehydrate the specimen. After 5 to 10 seconds, most of this liquid is drawn off. (6) When the remaining dioxane has evaporated almost entirely (evaporation to dryness does not harm the specimen if the next step is not delayed more than a few minutes), a drop of the resin is added and a cover slip applied. Air bubbles are less likely to form if a small drop of the mounting fluid is applied to the opposing surfaces of both the slide and the cover slip and the drops permitted to coalesce before the slip is lowered completely. Pressure on the cover slip will serve to flatten the preparation so that fungus structures are seen more readily.

This procedure, which is exceedingly simple and rapid, yields permanent preparations and produces slides of a quality equal to or better than those obtained by more complicated methods. The use of polyvinyl alcohol-lactophenol-cotton blue permits more rapid preparation of "permanent" mounts than does this new method, but *fungus* structures stained with the PVA medium lose their color quickly and cotton blue so applied penetrates clinical materials poorly. The original paper should be consulted for suggested modifications on the general procedure for different categories of clinical specimens and for additional applications.—*J. A. Kennedy.*

MACKLIN, CHARLES C. **Mouse epicytes and their intramural processes.** *Canad. J. Res., Sec. D,* **28,** *5–13.* 1950.

The epicytes from mouse lungs were studied by the following method: Etherize animals, open the abdomen, cut the great vessels, and nick the diaphragm to collapse the lungs; fill the lungs to moderate inflation by a hypodermic syringe with the needle inserted in the trachea; tie the trachea; immerse the lungs, heart, and great vessels in a fixing fluid such as Regaud's or 10% neutral formalin; after the lung becomes stiff, cut it into pieces; leave pieces in the fixing fluid; block mordant in 3% aqueous potassium bichromate or section mordant in 5% iron alum for as long as 7 days; stain in 0.01% to 0.1% aqueous hematoxylin 3 to 7 days; differentiate in iron alum, 5% diminishing to 2%, or in a saturated solution of picric acid; counterstain in orange G or fast green FCF. The epicytes are described.—*H. P. Riley.*

SULKIN, NORMAN M., and KUNTZ, ALBERT. **Histochemical determination of ribose nucleic acid in vertebrate tissues following extraction with perchloric acid.** *Proc. Soc. Exp. Biol. and Med.,* **73,** *413–5.* 1950.

Sensory and autonomic ganglia, and glandular tissues from rats and cats were fixed 8–15 hr. in Zenker's fluid and washed in running water for 2 hr. One half of each block was soaked in 10% perchloric acid at 5° C. for 18 hr. while the other half served as a control. The treated blocks were washed for 2 hr. in running water, and both these and the controls embedded in paraffin. A similar series of treated and control blocks were prepared without embedding and sectioned by the freezing technic. After rinsing in water, the frozen sections were stained with 1% toluidine blue for 5 min., counterstained with 0.5% alcoholic eosin (ethyl eosin?) for 15 sec., differentiated in 95% alcohol and mounted in glycerol. Paraffin sections were stained by Mallory's phloxine-methylene-blue method. The results of the experiments indicate that perchloric acid may be substituted for ribonuclease in the histochemical determination of ribonucleic acid. Frozen sections gave more consistent results than paraffin sections.—*H. A. Davenport.*

MICROÖRGANISMS

KNAYSI, G., HILLIER, J., and FABRICANT, C. **The cytology of an avian strain of *Mycobacterium tuberculosis* studied with the electron and light microscope.** *J. Bact.*, **60,** *423–47.* 1950.

For this excellent comparative study, the Sheard strain of *M. tuberculosis* was grown on collodion film on the surface of nutrient agar. The film was mounted on slides for light, and on screen for electron microscopy. For light microscopy the slides were prepared as follows: Giemsa – Fix air-dried slides 30 min. in 95% alcohol; stain 10 min. in Giemsa's solution diluted 5 times with distilled water; hydrolysis is not necessary; other dyes can be used in the same way. Sudan III – Drain and blot water from film on cover glass; invert it on a drop of saturated Sudan III in 75% alcohol. Sudan black B in ethylene glycol – Float the collodion film on water at 80° C. before mounting it on the cover slip as for Sudan III. Desoxyribonuclease – Float collodion cultures on water at 80° C. 10 min.; pick up on 22 × 40 mm. cover glass; air dry; place in enzyme solution – 5 mg. crystalline enzyme (Worthington Chem. Co., Freehold, N. J.) to 100 ml. distilled water adjusted to pH 6.8 with neutral broth; incubate at 45° C. ½–1 hr.; use a control of distilled water and neutral broth.—*Virgene Kavanagh.*

KLIGMAN, A. M., and MESCON, H. **The periodic-acid-Schiff stain for the demonstration of fungi in animal tissue.** *J. Bact.*, **60,** *415–21.* 1950.

The periodic-acid-Schiff stain which colors cellulose and chitin strongly forms the basis for this method of showing the distribution of many types of fungi in skin and internal organs much more clearly than most methods that have been tried. The procedure (Hotchkiss-McManus) is as follows: Fix in 4% formaldehyde or Rossman's fluid (or others); dehydrate; embed; section; remove paraffin and pass to absolute alcohol; wash in distilled water; place in 1% periodic acid for 5 min.; wash 15 min. in running tap water; strain 10–15 min. in Schiff reagent; transfer directly to two changes of either of the following solutions for 5 min. each: (1) 5 ml. 10% potassium metabisulfite, 5 ml. 1 *N* HCl, 100 ml. water, or (2) 5 ml. thionyl chloride, 100 ml. distilled water; wash 10 min. in running tap water; counterstain with light green; dehydrate; clear; and mount. Schiff reagent may be prepared as follows: Dissolve 0.5 g. basic fuchsin with 100 ml. boiling distilled water; cool to 50° C.; filter; (1) add 10 ml. 1 *N* HCl and 0.5 g. anyhydrous potassium metabisulfite or (2) add slowly 5–8 ml. thionyl chloride; stand in dark overnight; if darker than pale straw, decolorize by shaking with 0.25–0.50 g. charcoal and filtering immediately.—*Virgine Kavanagh.*

LAMANNA, C., and MALLETTE, M. F. **The relation of the Gram stain to the cell wall and the ribonucleic acid content of the cell.** *J. Bact.*, **60,** *499–505.* 1950.

Dyar's method (J. Bact., **53,** 498) of staining cell walls with Congo red following a mordant in cetyl pyridinium chloride was used after Gram staining (Burke, J. Bact., **7,** 159) without counterstaining. Prolonged treatment with cetyl pyridinium chloride washed out the Gram stain from the walls of bacteria and yeasts, showing Gram-positive granules in the interior of the yeasts. By shaking live yeast with cholorform (45 g. yeast in 220 ml. distilled water and 6 g. NaCl to 150 ml. reagent chloroform) in a Waring blendor 5 min. as much as ⅓ of the ribonucleic acid was removed without affecting the gram positive material.—*Virgene Kavanagh.*

OLSON, J. C., Jr. and BLACK, L. A. **A comparative study of stains proposed for the direct microscopic examination of milk.** *Milk and Food Techn.*, **14,** *49–51, 64.* 1951.

A comparison was made in eight laboratories of results obtained with six staining solutions for the direct microscopic count. Great variation was observed

in the reports obtained; only two of the six solutions failed to give highest counts in at least one of the eight laboratories. Hence, the results are not very conclusive. So far as they are significant, they seem to favor the solution previously proposed by Levine and Black which contains no water or acid, and in which the methylene blue is dissolved in ethanol and sulfuric ether.—*H. J. Conn.*

RIDDELL, ROLAND W. **Permanent stained mycological preparations obtained by slide culture.** *Mycologia,* **42,** *265–70.* 1950.

Directions are given for preparing agar squares 1 cm. on the side and 2 mm. in thickness. All procedures are carried on with aseptic precautions. An agar square is transferred to a sterile slide and a needlepoint inoculum of spores, or a small bit of mycelium, is placed at the center of each edge of the agar block over the whole 2 mm. depth at this point. A sterile cover slip is placed upon the agar square and the slide placed in a moist chamber. When growth has extended for about 1 mm., the cover slip is lifted carefully, and inverted. The agar square is then carefully removed from the slide. All that remain on both slide and cover slip are four areas of growth joined by scanty lines of mycelium. A drop of 95% ethyl alcohol is applied to the center of each of the rings of growth and allowed to flow peripherally to wet the fungus. Just before it has completely evaporated, a drop of lactophenol blue is applied in similar fashion. A clean cover slip is applied to the slide preparation, and a clean slide is applied to the cover slip preparation. Lactophenol blue is prepared as follows: dissolve by gentle heating, phenol crystals 20 g., lactic acid 20 ml., glycerol 40 ml. and distilled water 20 ml. Then add 0.05 g. cotton blue (methyl blue). *(Note:* no certification number, nor dye percentage in the dry stain is given.) After cleaning, the edges of the cover slips are sealed to the slides by the use of colored nail lacquer.—*Elbert C. Cole.*

SECONDO, SANI. **Ricerche sul comportamento delle sostanze a gruppi –SH nei microorganismi.** *Bol. Soc. Ital Biol. Sp.,* **25,** *1152–3.* 1949.

Treatment with 10% trichloroacetic acid for 5 min., followed by treatment with 5% sodium nitroprusside for 5 min., then exposure to ammonia fumes, resulted in a red color, considered to demonstrate the existence of –SH groups, in all microörganisms tested.—*J. Dufrenoy.*

WACHSTEIN, M., and PISANO, M. **A new staining technique for polar bodies.** *J. Bact.,* **59,** *357–60.* 1950.

The polar bodies of *Corynebacterium Spp.* were clearly demonstrated by Gomori's method for acid phosphatase, but it was later observed that the enzyme was not necessary to obtain the reaction in these bodies. The method for depositing lead sulfide on the polar bodies of bacteria is as follows: Heat, fix and dry the bacterial film on a slide; place in 10% lead nitrate solution 15 min. at room temperature or flood slide and heat gently for 2 – 3 min.; rinse thoroughly with tap water; immerse 1 – 2 min. in 1% aqueous sulfurated KOH (keeps in refrigerator for several weeks) or in a fresh solution of approx. 1 ml. light ammonium sulfide in 50 ml. water; wash; counterstain 30–60 sec. with 1% aqueous safranine O. These polar bodies were not stained by the Feulgen technic.—*V. Kavanagh.*

WHITE, P. G., and WILSON, J. B. **Differentiation of smooth and nonsmooth colonies of Brucellae.** *J. Bact.,* **61,** *239–40.* 1951.

Variations in Brucella cultures may be demonstrated readily as follows: Pour and dry for 24 hr. plates of Albimi Brucella agar modified to contain 2.5% agar, 1% glucose, and 5% glycerol; streak plate with culture; incubate 96 hr. at 37° C.; flood plate with 1:2000 aqueous crystal violet for 15 sec.; decant excess dye into disinfectant; examine colonies under low power of dissecting microscope with obliquely transmitted light; colonies may be distinguished up to 96 hr. after staining. Smooth colonies are light blue-green with a peripheral ring deepening

to blue-violet in time. Nonsmooth colonies appear red to blue red and give three color reactions: rough, deep violet-red; typical mucoid, lighter blue-red; one mucoid *Brucells suis,* deep purple-blue. Safranin O. differentiates between smooth and nonsmooth colonies but does not show the variations among the nonsmooth types. None of the other dyes tested were of much use.—*Virgene Kavanagh.*

ZEBROWSKI, T. **(Comparative valuation of two methods for staining tubercle bacilli, Halberg's and Ziehl-Neelsen's.)** *Przeglad Lekarski,* **6,** *62–6.* 1950.

A smear of tuberculous sputum was stained first with the Ziehl-Neelsen method, and the number of microörganisms and the time of observation estimated. The same films were then treated with 95% ethanol for 5 min. to dissolve the oil and stained with night blue. Counterstaining was done with carbol-fuchsin. The same fields were examined, to estimate again the number of microörganisms and the time required. The tubercle bacilli appeared at least 3 times as numerous in fields stained with night blue as in those stained according to the Ziehl-Neelsen method, especially in the thicker preparations. Only half as long a time proved necessary to count the organisms. Moreover the tubercle bacilli seem to be larger, more strongly stained and stand out more distinctly against the background. Much time in laboratory routine is saved by using the Hallberg method. *Author abstract.* (Courtesy of Biological Abstracts).